

Proceedings
of the
Society
for
Experimental Biology and Medicine

VOL. 68

JULY-AUGUST, 1948

No. 3

SECTION MEETINGS

SOUTHWESTERN

University of Texas Med. School

May 7, 1948

SOUTHERN CALIFORNIA

University of California, Los Angeles

June 10, 1948

16504

Mechanism of Pyrogen Induced Fever.*

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The fever which occurs in several animal species following the injection of certain bacterial products¹⁻³ has been observed to be associated with an increase in oxygen consumption.⁴⁻⁸ Quantitative study of heat exchange in man during this "pyrogenic reaction" has revealed that during the "chill phase" there is a substantial increase in heat production with no change in heat loss.⁴ Simulated shivering in a normal man has been shown to produce the same magnitude of increase in heat production as observed during

the pyrogenic chill.⁹ However, in contrast to the pyrogenic reaction, a simultaneous increase in heat loss was observed, so that only a slight fever developed. It may thus be inferred that interference with heat loss is an important factor in the production of pyrogenic fever, and that demonstration of its alteration during this reaction is masked by the appearance of increased heat production due to activity of the skeletal muscles in the process of shivering. Consequently, it is of interest to know if a febrile response to pyrogen can occur in an animal incapable of increased heat production by voluntary muscle movement, and if so, to determine under these conditions the nature of the alteration in heat exchange. Thus, the pyrogenic reaction has been studied in the curarized dog.

The pyrogenic substance employed in the present studies is a purified material obtained from *Pseudomonas aeruginosa*[†] and provided

* Aided by a grant from Baxter Laboratories, Inc., Morton Grove, Ill.

¹ Seibert, F. B., *Am. J. Physiol.*, 1924, **71**, 621.

² Ranson, S. W., Jr., *Arch. Int. Med.*, 1938, **61**, 285.

³ Co Tui, D. Hope, Schrift, M. H., and Powers, J., *J. Lab. and Clin. Med.*, 1944, **29**, 58.

⁴ Barr, D. P., Cecil, R. L., and DuBois, E. F., *Arch. Int. Med.*, 1922, **29**, 608.

⁵ Grollman, A., *J. Clin. Invest.*, 1929, **8**, 25.

⁶ Leimdorfer, A., *Biochem. Z.*, 1932, **133**, 409.

⁷ Altschule, M. D., Freedberg, A. S., and McManus, M. J., *J. Clin. Invest.*, 1945, **24**, 878.

⁸ Hildenbrandt, F., *Arch. f. Exp. Path. u. Pharmacol.*, 1943, **201**, 278.

⁹ DuBois, E. F., *Basal Metabolism in Health and Disease*, Lea and Febiger, Philadelphia, 1927.

[†] Chemical properties of this substance will be described in a forthcoming paper by Dr. L. G. Ginger and coworkers.

TABLE I.
Influence of Pyrogen on Heat Exchange in Curarized Dogs.

Exp. No.	Wt, kg	Avg pre-febrile heat production and loss (cal./min.)	Period of rising temperature				Fraction of stored heat due to alteration of:	
			Duration (min.)	Temp. increment (°C)	Change in avg		Heat loss (%)	Heat prod. (%)
					Heat loss (%)	Heat prod. (%)		
1	7.3	.210	120	1.52	-29.0	+ 7.6	79.2	20.8
2	8.2	.254	135	1.28	-21.3	+ 3.0	87.6	12.4
3	10.5	.306	166	2.86	-33.7	+15.4	68.7	31.3
4	10.5	.424	170	1.40	-14.6	+ 4.0	77.9	22.1
5	8.2	.250	110	1.44	-29.6	+ 6.0	83.2	16.8
6	6.8	.253	140	1.34	-16.6	+ 4.7	77.8	22.2
7	14.5	.485	70	1.12	-35.9	+ 3.9	90.1	9.9
8	11.4	.335	240	2.35	-14.3	+13.4	51.4	48.6
9	6.3	.250	80	0.84	-13.2	+ 8.8	59.9	40.1
10	10.1	.306	100	1.66	-41.5	+ 3.9	91.4	8.6
11	11.1	.426	130	2.84	-25.8	+21.4	54.8	45.2
12	11.8	.491	110	1.50	-22.6	+ 4.7	82.8	17.2
13	10.0	.291	180	1.90	-22.7	+ 7.6	74.9	25.1
14	15.0	.491	200	1.55	-13.6	+ 5.1	74.0	26.0
Mean					-23.9	+ 7.8	75.3	24.7
Stand. Dev.					± 9.0	± 5.0	±12.0	±12.0

for us[†] in the form of a standard concentrate. This material will produce, on first intravenous injection, a febrile response in the normal dog in doses of 2-3 μ g/kg. In doses of 25-50 μ g/kg this material produced, after a latent period of 18.8 ± 7 minutes, a rise in rectal temperature of $1.77 \pm 0.5^\circ\text{C}$ in 14 non-curarized dogs.

For the studies performed on the curarized dog, crystalline *d*-tubocurarine[§] was employed. The initial paralyzing dose was 1-1.5 mg/kg given intravenously, and subsequent curarization was maintained by the hourly intramuscular injection of 0.5 mg/kg. Artificial respiration was maintained in these animals by means of a Starling-Palmer pump adjusted to give a ventilation-oxygen consumption ratio of approximately 20. In order to insure a relatively painless and yet airtight connection between the dog and an oxygen filled spirometer, an endotracheal airway with an inflatable cuff was employed. Carbon dioxide was removed from this closed system by soda-lime and water vapor by calcium chloride. To insure that inspired air was relatively dry, light

weight oil, rather than water, was employed in the spirometer. Oxygen consumption from the spirometer was continuously measured. Continuous rectal temperatures were obtained by means of a recording resistance thermometer.

With room temperature held at $27 \pm 1^\circ\text{C}$ these curarized animals maintain a very constant rectal temperature in the neighborhood of $38.5\text{-}39^\circ\text{C}$ and a very constant oxygen consumption at an average of 70 ± 21 cc per minute.

After a control period of 1-2 hours, the

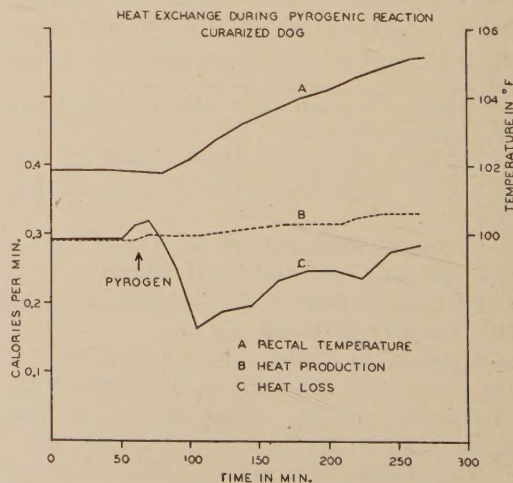


FIG. 1.

[†] Through the courtesy of Dr. N. M. Nasset of Baxter Laboratories.

[§] Through the courtesy of Dr. R. K. Richards of Abbott Laboratories.

TABLE II.
 Time of Occurrence and Magnitude of Maximum Alteration of Heat Exchange.

Exp. No.	Duration of period of:		Maximum alteration of heat exchange			
	Latency* (min.)	Rising temp. (min.)	Time of occurrence†		Magnitude‡	
			Heat loss (min.)	Heat prod. (min.)	Heat loss (%)	Heat prod. (%)
1	14	120	20	65	-54	+10
2	15	135	57	68	-28	+15
3	7	166	30	122	-69	+30
4	20	170	23	98	-43	+9
8	25	240	17	197	-63	+19
11	15	130	30	130	-74	+38
12	18	110	22	82	-37	+6
13	12	180	10	150	-44	+11
14	21	200	40	160	-21	+9
Mean	16.3	161.2	27.7	119.1	-48.1	+16.3
Stand. Dev.	±5	±42	±14	±45	±18	±11

* Time from injection of pyrogen to onset of fever.

† Time from onset of fever.

‡ Per cent of pre-febrile control level.

curarized dogs were injected intravenously with 25-50 $\mu\text{g}/\text{kg}$ of the standard pyrogenic material. After an average latent period of 16.3 ± 5 minutes, the rectal temperature began to rise, and over a period of 161.2 ± 42 minutes had risen $1.69 \pm 0.6^\circ\text{C}$. It is thus apparent that pyrogen induced fever is essentially unaltered by prior curarization and that the reaction can occur in the absence of increased heat production due to movement of the voluntary muscles.

The continuous measurement of rectal temperature and oxygen consumption in these animals makes possible continuous estimation of heat production and loss. The dogs were in a fasting state (24 hours) and the observed R.Q. was of such magnitude (approximately 0.80) as to indicate the use of the factor 4.8 as the caloric equivalent of 1 liter of oxygen. By means of this factor, heat production was determined from oxygen consumption during the pre-febrile control period and during the period of rising temperature.

Heat loss, while equal to heat production during the pre-febrile period of constant temperature, becomes equal to heat production minus heat storage during the period of rising temperature. Heat storage is the product of the rise in body temperature and the specific heat of tissue. The accepted figure for specific heat of tissue, 0.83,⁹ has been employed

to calculate heat storage, and this in turn to calculate heat loss. In this fashion heat loss and heat production have been continuously determined.

It may be observed (Table I) that during the period of rising temperature, following the injection of pyrogen, the curarized dog shows an increase in average heat production of $7.8 \pm 5\%$ over the pre-febrile control level. The heat stored during this period exceeded the heat produced, indicating that a reduction in heat loss had occurred. The average heat loss during the period of rising temperature was found to be $23.9 \pm 9\%$ less than during the pre-febrile control period. In these animals, therefore, the average fraction of the stored heat due to increased heat production was $24.7 \pm 12\%$ while the fraction due to decreased heat loss was $75.3 \pm 12\%$. Thus, the major fraction of the febrile response to pyrogen is, under these conditions, one of reduction in heat loss.

Additional information as to the relative importance of decreased heat loss and increased heat production during the pyrogenic reaction is obtained from consideration of the time of occurrence of these changes in relation to the onset of fever. It may be seen (Fig. 1) that reduction in heat loss corresponds with the onset of the fever and occurs at a time when heat production is essentially unchanged.

The maximum reduction of heat loss occurs early in the course of the fever and heat loss then slowly returns toward the pre-febrile level as the temperature increases. However, the increase in heat production is progressive and is associated with the rise in temperature. The maximum increase in heat production does not occur until near the peak of the rise in temperature.

Data relative to the time of occurrence and magnitude of the maximum alteration of heat exchange is shown (Table II). The average time of occurrence of the maximum reduction of heat loss was 27.7 ± 14 minutes after the onset of the rise in temperature, while the maximum increase in heat production occurred 119.1 ± 45 minutes after the onset of fever. The degree of alteration of heat exchange which occurred in a rather brief period of time is revealed by the magnitude of the maximum reduction in heat loss, the average of which was $48.1 \pm 18\%$ below the pre-febrile control level of heat loss. The corresponding elevation of heat production was $16.3 \pm 11\%$ above the pre-febrile level.

It is concluded that the mechanism of the pyrogenic reaction is such that it can occur in the complete absence of increased heat production due to activity of the voluntary muscles, as in the act of shivering, and that under these conditions the primary disturbance is one of rapid and marked reduction of heat loss. The observed increase in heat production is of unknown origin. However, the correlation between the per cent increase in average heat production and the increment in

rectal temperature is good (correlation coefficient = 0.82) and the regression is such as to indicate that there is a 7.11% increase in average heat production per degree centigrade rise in rectal temperature. It may, thus, be assumed that the increase in heat production under these conditions is due to the increase in temperature, a phenomenon previously described.¹⁰

It is known that peripheral vasoconstriction occurs coincident with the onset of the pyrogenic reaction.^{2,7,11-13} Since the curarized dog, when artificially ventilated, cannot alter heat loss by reducing the rate of evaporation of water from the lungs or alter radiating surface by change in position, it may be presumed that vasoconstriction is responsible for the observed reduction in heat loss.

Summary. Typical pyrogen induced fever occurs under conditions in which increased heat production due to activity of skeletal muscles is not possible. Under these conditions the mechanism of the pyrogenic reaction is one of sudden and marked reduction of heat loss. The slight increase in heat production under these conditions is correlated with the rise in temperature and is assumed to be a consequence rather than a cause of the fever.

¹⁰ Dubois, E. F., *J. Am. Med. Assn.*, 1921, **77**, 352.

¹¹ Fremont-Smith, F., Morrison, R. L., and Makepeace, A. W., *J. Clin. Invest.*, 1929, **7**, 489.

¹² Pinkston, J. O., *Am. J. Physiol.*, 1934, **110**, 448.

¹³ Johnson, C. A., Scupham, G. W., and Gilbert, N. C., *Surg., Gyn. and Obs.*, 1932, **55**, 737.

Effect of Dibenamine on Renal Hypertension in Rats.*

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(Introduced by Louis S. Goodman.)

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The sequence of events by which interference with renal hemodynamics leads to elevation of the systemic blood pressure has been carefully studied (Braun-Menendez, *et al.*,¹ and Goldblatt²) and has been shown to be independent of nervous mechanisms. However, scattered reports³⁻⁵ have suggested that drugs acting to block the vasopressor action of the sympathetic nervous system may lower the blood pressure in animals with renal hypertension. Sympathectomy also reduces the blood pressure in animals with renal hypertension,⁶ but the reduction is limited in extent and prior sympathectomy does not prevent the development of renal hypertension.⁶⁻⁸

The use of drugs in the study of neurogenic factors in renal hypertension has been limited by the relative ineffectiveness of the available agents. Demonstration of the high potency and specificity of the adrenergic blocking

action of N,N-dibenzyl- β -chloroethylamine (Dibenamine)⁹ suggested that it would be a useful tool in evaluating the role of the sympathetic nervous system in both experimental renal hypertension and human essential hypertension. The present study is concerned with the response of rats with renal hypertension to Dibenamine.

Methods. Mature, 200-300 g, Sprague-Dawley strain rats were made hypertensive by bilateral compression of the renal parenchyma according to the method of Grollman.¹⁰ The procedure was carried out in two stages 4 to 6 weeks apart. The incidence of satisfactory hypertension obtained was never more than 30%.

Blood pressure was measured by a modification of the tail plethysmograph method of Byrom and Wilson,¹¹ using unanesthetized animals and a thin rubber plethysmograph membrane around the tail. A 10 mm occlusion cuff was employed and the plethysmograph was maintained at 42-44°C. This temperature provides adequate vasodilatation and obviates the necessity of heating the entire animal.¹² The rats were housed at a nearly constant temperature and blood pressure readings were made in the same room.

Dibenamine and its alcohol derivative, N,N-dibenzyl-ethanolamine, were administered orally in small lactose tablets. Oral administration was employed in order to avoid the difficulty of repeated intravenous injections and the local tissue damage which follows

* This investigation was aided by a grant from Givaudan-Delawanna, Inc. The Dibenamine and N,N-dibenzyl-ethanolamine were kindly supplied by Dr. William Gump of this company.

¹ Braun-Menéndez, E., Fasciolo, J. C., Leloir, L. F., Muñoz, J. M., and Taquini, A. C., *Renal Hypertension*, 1946, Charles Thomas, publisher, Springfield, Ill.

² Goldblatt, H., *Physiol. Rev.*, 1947, **27**, 120.

³ Jacobs, J., and Yonkman, F. F., *J. Lab. and Clin. Med.*, 1944, **29**, 1217.

⁴ Reed, R. K., Sapirstein, L. A., Southard, F. D., and Ogden, E., *Am. J. Physiol.*, 1944, **141**, 707.

⁵ Sapirstein, L. A., and Reed, R. K., *Proc. Soc. Exp. Biol. and Med.*, 1944, **57**, 135.

⁶ Alpert, L. K., Alving, A. S., and Grimson, K. S., *Proc. Soc. Exp. Biol. and Med.*, 1937, **37**, 1.

⁷ Freeman, N. E., and Page, I. H., *Am. Heart J.*, 1937, **14**, 405.

⁸ Verney, E. B., and Vogt, M., *Quart. J. Exp. Physiol.*, 1938, **28**, 253.

⁹ Nickerson, M., and Goodman, L. S., *J. Pharm. and Exp. Therap.*, 1947, **89**, 167.

¹⁰ Grollman, A., *Proc. Soc. Exp. Biol. and Med.*, 1944, **57**, 102.

¹¹ Byrom, F. B., and Wilson, C., *J. Physiol.*, 1938, **93**, 301.

¹² Sobin, S. S., *Am. J. Physiol.*, 1946, **146**, 179.

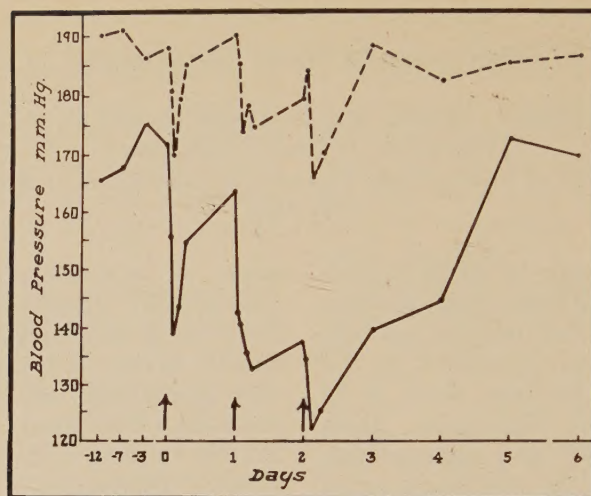


FIG. 1.

Blood pressure responses of renal hypertensive rats to Dibenamine; (—•—•—) average of 11 animals showing a cumulative effect and (---•---) average of 6 animals with no cumulative effect. Arrows indicate oral administration of 100 mg/kg Dibenamine.

subcutaneous or intraperitoneal administration of the drug.⁹

A series of experiments in which the carotid blood pressure of rats under pentobarbital anesthesia was recorded directly demonstrated that a single oral dose of 100 mg/kg Dibenamine largely blocked and reversed the pressor response to small (1-2.5 μ g/kg) intravenous injections of epinephrine but not the response to large doses. The incomplete adrenergic blocking action of this single oral dose of Dibenamine reached a maximum about 2 hours after administration and repeated daily administrations were found to have a moderate cumulative effect.

Results. Seventeen hypertensive rats were given oral doses of 100 mg/kg Dibenamine daily for 3 days and their blood pressure determined at frequent intervals for 6 days. The results of these experiments are graphed in Fig. 1. Two reasonably distinct types of response were observed and have been plotted separately. A total of 11 animals (65%) developed a progressive lowering of the blood pressure to approximately normotensive levels. In this group the average blood pressure did not return to pretreatment levels until 3 days after the last administration of Dibenamine.

An early fall in pressure reaching a maximum in 2 to 6 hours occurred after each dose of Dibenamine. The maximum depressor effect tended to occur earlier after the first dose than after subsequent administrations.

The second group consists of 6 animals (35%) in which the blood pressure returned to within 20 mm Hg of the control value in less than 24 hours after each Dibenamine administration. A transient depressor action was noted after each dose of Dibenamine, but there was little or no cumulative effect. The immediate depressor action was also smaller in this group than in the group reacting with a more sustained fall in blood pressure. These animals had a somewhat higher average blood pressure prior to treatment than those which responded with a sustained reduction, but the significance of this factor is difficult to evaluate.

For comparative purposes the 17 hypertensive animals described above may be divided into two groups on the basis of duration of hypertension. Animals which had been hypertensive (blood pressure greater than 160 mm Hg) for over 2 months (5 rats) were considered as late hypertensives, and those hypertensive for less than 2 months (12 rats) were

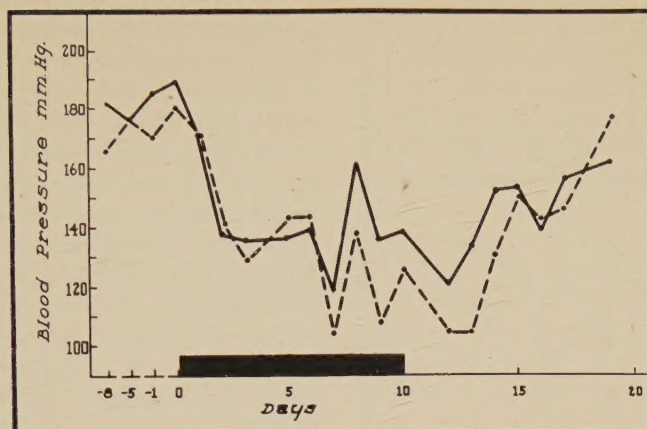


FIG. 2.

Blood pressure responses of renal hypertensive rats to daily oral administration of 100 mg/kg Dibenamine; (—) average of 5 animals and (---) a representative individual. Period of Dibenamine administration indicated by black bar.

considered as early hypertensives. Two (40%) of the former and 9 (75%) of the latter developed a sustained lowering of the blood pressure with Dibenamine.

More prolonged experiments were carried out on 5 hypertensive rats previously shown to respond well to a single dose of Dibenamine. Single 100 mg/kg oral doses of the drug were given daily for 10 days. The blood pressure was determined just before each administration and for 10 days following the end of treatment. Averaged data from the 5 animals and the record of a representative individual are presented in Fig. 2. Although considerable fluctuation of the blood pressure occurred during the period of Dibenamine administration, the average pressure was lowered

into the normal range for most of the period of treatment and for 3 to 4 days after the end of medication.

It is of interest that the blood pressures of all rats in this group tended to fluctuate together, indicating that the pressure in these animals was still capable of responding to environmental factors to at least a limited extent. The effect of external stimuli was confirmed by tests performed on the fifth day of Dibenamine administration. Two blood pressure readings were taken on this day—one the usual morning determination, the second while the animals were in a severely cramped position caused by moving the partitions in the round wire cages in which they were held during blood pressure determinations. The results of these readings are shown in Table I. In all cases the blood pressure was somewhat elevated by the factor of an environmental stress.

TABLE I.
Effect of Environmental Stress on Blood Pressure of Renal Hypertensive Rats Treated with Dibenamine.

Animal	Blood pressure	
	Normal reading	While cramped in cage
1	130	140
2	143	154
3	134	160
4	122	132
5	150	172
Avg	136	152

To determine whether the observed reductions in blood pressure were due to the adrenergic blocking action of Dibenamine, hypertensive rats were administered N,N-dibenzyl-ethanolamine, which differs from Dibenamine only in having the β -chlorine of the latter replaced by a hydroxyl group. Dibenzyl-ethanolamine is probably the compound to which Dibenamine is transformed in the

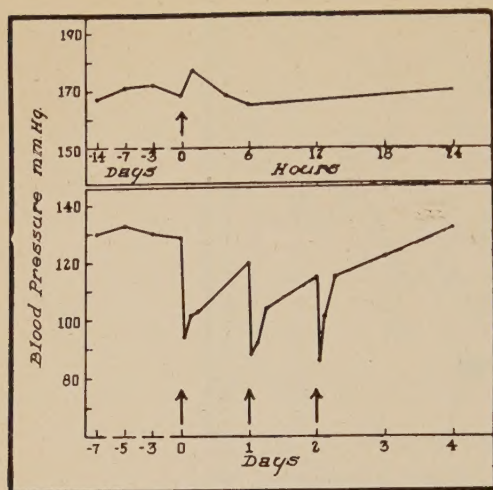


FIG. 3.

Upper record: Average blood pressure response of 5 renal hypertensive rats to a single oral administration of 300 mg/kg N,N-dibenzyl-ethanolamine given at the arrow.

Lower record: Average blood pressure response of 6 normotensive rats to Dibenamine. Arrows indicate oral administration of 100 mg/kg Dibenamine.

body;¹³ it has many pharmacological properties in common with Dibenamine, but has no demonstrable adrenergic blocking action. A single 300 mg/kg oral dose of this compound caused no reduction in blood pressure in 5 hypertensive rats (Fig. 3, upper).

Six rats which had been operated upon for compression of the kidneys, but which had not become hypertensive, were also administered 100 mg/kg doses of Dibenamine orally, with the results presented in Fig. 3 (bottom). Each administration of Dibenamine caused a transient lowering of the blood pressure, but no significant sustained reduction was induced.

In experiments on anesthetized cats the action of Dibenamine was found to be limited almost entirely to blocking excitatory effects of sympathetic nerve stimulation or sympathomimetic amines.¹⁴ It does not significantly alter the pressor response to angiotonin† (Fig. 4).

¹³ Nickerson, M., and Gump, W., to be published.

¹⁴ Nickerson, M., and Nomaguchi, G. M., *J. Pharmacol. and Exp. Therap.*, 1948, **93**, 40.

† Kindly supplied by Drs. Helmer and Koehlstadt of the Lilly Laboratory for Clinical Research, Indianapolis, Ind.

No significant side-effects of Dibenamine administration were noted in either the normotensive or hypertensive animals.

Discussion. The data presented above indicate that Dibenamine causes a decrease in the systemic arterial pressure of both normal and renal hypertensive rats. A hypostatic factor is probably involved in this hypotension inasmuch as the reductions obtained are considerably greater than are observed when Dibenamine is administered to anesthetized animals or normal recumbent humans.^{9,15} The depressor effect is apparently dependent upon the adrenergic blocking action of the drug.

The Dibenamine-induced blood pressure reduction was greater and more sustained in most of the renal hypertensive animals than in the normotensives, but this does not necessarily imply that the difference represents increased sympathetic pressor activity. In the absence of stress, the blood pressure can be

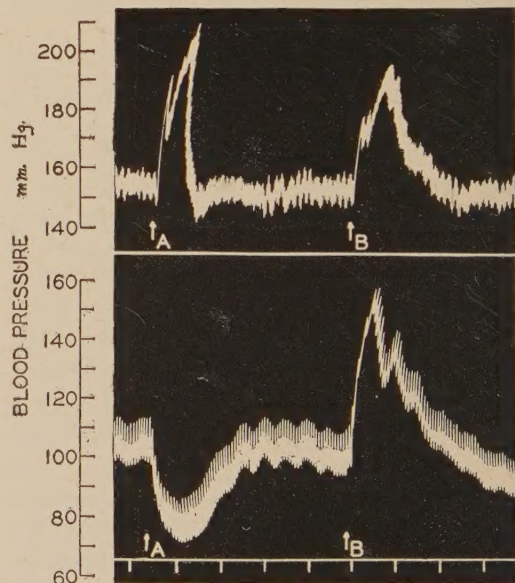


FIG. 4.

Blood pressure responses of cat to 1.5 µg/kg epinephrine (A) and 2 cat units angiotonin (B). Upper records before and lower records 45 minutes after the intravenous administration of 10 mg/kg Dibenamine. The apparent potentiation of angiotonin is due to the lowered initial pressure. Pentobarbital anesthesia. Time in minutes.

¹⁵ Haimovici, H., and Medinets, H. E., *Proc. Soc. Exp. Biol. and Med.*, 1948, **67**, 163.

maintained within normal limits even in the complete absence of sympathetic nerve impulses. After complete sympathectomy in normal animals the blood pressure returns to essentially normotensive levels,¹⁶⁻¹⁷ and a similar but less conspicuous adjustment appears to occur after sympathetic blockade with Dibenamine.⁹ The possibility must therefore be considered that the ability of the vascular system to compensate for the absence of sympathetic impulses rather than the level of sympathetic activity may be of primary importance in determining the extent of the depressor response to sympathetic blockade. This possibility gains added weight when it is considered that the major difference between the responses of normal and renal hypertensive animals to Dibenamine is the much slower rate at which the blood pressure of the latter returns to pretreatment levels.

It is somewhat difficult to compare the results of this study with reports covering the use of other sympathetic blocking agents in experimental renal hypertension. Unfortunately, the work of Reed and coworkers⁴ and Sapirstein and Reed⁵ on the effects of yohimbine and 883F, respectively, on renal hypertension in rats involved only acute blood pressure changes following single injections of the drugs and the extent to which the recorded blood pressure changes were due to specific adrenergic blockade is difficult to evaluate. Bing and Thomas¹⁸ observed no depressor action of single injections of 883F in dogs with chronic renal hypertension.

Jacobs and Yonkman,³ and Wilburn *et al.*¹⁹ studied the ability of yohimbine and Dibenamine, respectively, to produce a sustained lowering of the blood pressure of renal hyper-

tensive dogs. In the latter report, the results were correlated with studies of the adrenergic blocking action of the drug. Both groups found some reduction in blood pressure, but were unable consistently to return the pressure to normotensive levels. In both studies the average reduction was somewhat less than observed in the present experiments on rats. As in the work reported above, they found wide variations in the response of different animals, and considerable fluctuations in blood pressure during the period of treatment.

None of the work reported to date allows a clear evaluation of the contention^{4,5,20} that the sympatho-adrenal system is of importance in late but not in early renal hypertension. Although the number of animals in the experiments described above is not adequate to provide conclusive evidence on this point, the fact that a lower percentage of animals with hypertension of over 2 months' duration responded to Dibenamine with a sustained lowering of blood pressure does not support this view.

Summary. 1. Oral administration of 100 mg/kg Dibenamine causes some reduction of blood pressure in both normotensive and renal hypertensive rats. The fall is usually greater and more prolonged in hypertensive animals. 2. The fall in blood pressure is due to the adrenergic blocking action of Dibenamine and does not follow the administration of larger doses of the adrenergically inactive N,N-dibenzyl-ethanolamine. 3. In 65% of the hypertensive animals tested, repeated daily doses of Dibenamine produced a persistent, but somewhat variable lowering of the blood pressure. The pressure usually returned to pretreatment levels in 3 to 4 days after the last administration of Dibenamine. 4. The bearing of these data on postulated neurogenic factors in experimental renal hypertension is discussed, and it is concluded that they do not support the contention that a greater neurogenic factor is involved in late than in early renal hypertension.

¹⁶ Cannon, W. B., Newton, H. F., Bright, E. M., Menkin, V., and Moore, R. M., *Am. J. Physiol.*, 1929, **89**, 84.

¹⁷ Grimson, K. S., Wilson, H., and Phemister, D. B., *Ann. Surgery*, 1937, **106**, 801.

¹⁸ Bing, R. J., and Thomas, C. B., *J. Pharmacol. and Exp. Therap.*, 1945, **83**, 21.

¹⁹ Wilburne, M., Katz, L. N., Rodbard, S., and Surtshin, A., *J. Pharmacol. and Exp. Therap.*, 1947, **90**, 215.

²⁰ Ogden, E., *Bull. N. Y. Acad. Med.*, 1947, **23**, 643.

Identification of Histamine in Blood by Paper Chromatography.

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Many experiments have demonstrated that the substance responsible for anaphylactic shock appears in the circulation during this reaction. On the basis of its biological activity, this substance has been identified as histamine. Attempts to provide chemical proof that this substance is histamine have failed because of the small amounts present, the large amounts of interfering substances and the non-specificity of the color reactions generally employed.

In the present study these difficulties have been overcome by a simple purification procedure combined with paper chromatography recently developed for the identification and separation of minute quantities of amino acids¹ and since applied to the identification of a variety of substances.

Method. Paper chromatography consists of the deposition of very small amounts of solution (*i.e.* 0.01 ml) containing a few micrograms of the substances to be separated and identified on a limited area of a filter paper strip. The edge of the strip closest to the point of application of the solution is then suspended from a trough containing a mixture of solvents, one of which is usually water and the other some immiscible liquid, such as phenol, collidine, butanol, etc. The solvent mixture moves down the filter paper strip by capillary action and carries with it the different compounds applied, each at a different rate, presumably according to its individual solubilities in each of the liquids of the solvent mixture. This affords a very effective method of separation and identification of the various components applied to the strip. In adapting these principles to the identification of histamine some modifications have been made. The most important is a procedure for the

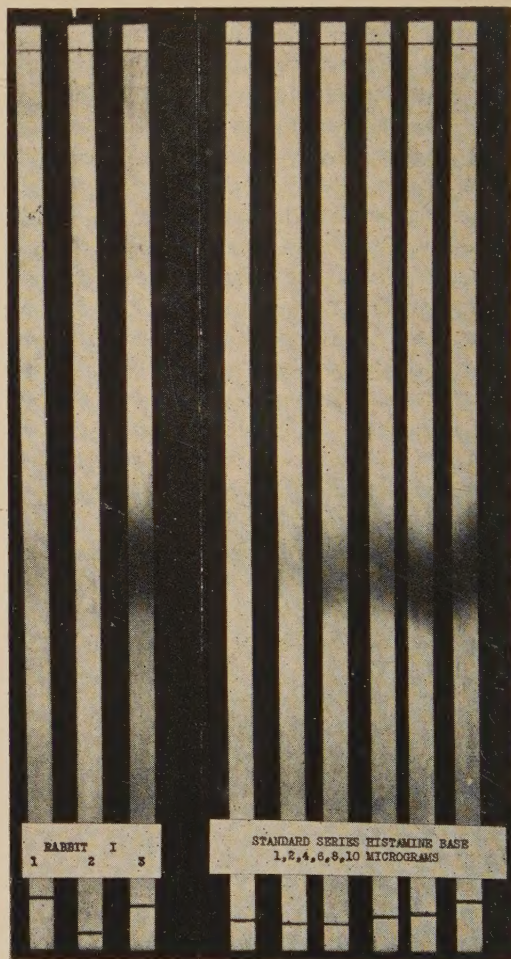


FIG. 1.
Chromatographic Evidence of Histamine Release in Anaphylaxis.

Left to right: (1) Chromatogram of plasma separated from blood of sensitized rabbit. (2) Chromatogram of plasma separated from blood of sensitized rabbit—antigen added to plasma *after* separation. (3) Chromatogram of plasma separated from blood of sensitized rabbit—antigen added to whole blood *before* separation of plasma. (4-9) Chromatograms of graded quantities of histamine.

Upper line—point of application to filter paper strip of butanol extract.

Lower line—point to which solvent mixture advanced at 15 hours.

* Rockefeller Foundation Fellow.

¹ Consden, R., Gordon, A. H., and Martin, A. J. P., *Biochem. J.*, 1944, **38**, 224.

application of a relatively large volume of solution containing the histamine to a restricted area of the filter paper strip. For this purpose capillary pipettes with very slow flow rate[†] in contact with the strips have been employed. The solvent as applied from the pipette is continuously evaporated at the same rate as it is deposited (approximately 1 ml per hour) by means of a hot plate with adjustable temperature control.

In the actual procedure 2-20 ml of plasma are diluted with an equal amount of water and the pH and salt concentration are adjusted according to the procedure used by McIntire² for the extraction of histamine with butanol. The histamine is then extracted from the butanol with a small amount of 0.5 N sulfuric acid which is, in turn, brought to the proper salt concentration and pH and re-extracted with approximately 3 ml of butanol. The final butanol extract is then applied to the filter paper strip by means of the previously described pipette. Control strips with known amounts of histamine are prepared in a similar manner. A set of strips is then suspended in a closed chamber from a trough containing butanol saturated with 10% ammonium hydroxide as the solvent mixture. After 12-15 hours the strips are removed, dried and treated with an alkaline diazotized solution of p-bromoaniline employed as a color

reagent for histamine by Baraud.³ Histamine, in excess of 1 μ g can be detected as a red band on the strips. Its presence and identity are established by the color of the band and more critically by the position of the colored band on the strips (R_f).^{†,1}

Results. The method described has been applied to prove the appearance of histamine in plasma when antigen is added (*in vitro*) to the blood of sensitized rabbits. Fig. 1 shows the results of such an experiment. Approximate estimation of the amounts of histamine released is possible by comparison with a standard series of strips (Fig. 1). Histamine has also been identified by this method in the blood of anesthetized dogs undergoing anaphylactic shock. In all experiments simultaneous biological assay of plasma extracts[§] on guinea pig ileum showed good agreement with the amounts of histamine found by paper chromatography.

Summary. A method for the chemical identification of histamine in blood has been described and used to prove the appearance of histamine in plasma after addition of antigen (*in vitro*) to the blood of sensitized rabbits and in the blood of dogs undergoing anaphylactic shock.

We wish to acknowledge the interest and helpful suggestions of Dr. Carl A. Dragstedt.

³ Baraud, J., Genevois, L., Mandillon, G., and Ringenbach, G., *C. R. Acad. Sci.*, 1946, **222**, 760.

[†] R_f is defined as the ratio of the distance traveled by the substance to the distance traveled by the solvent mixture.

[§] Purified by the cotton succinate method, see reference 2.

[†] Kindly suggested and made by J. J. Svarz, Department of Chemistry, Northwestern University Dental School.

² McIntire, F. C., Roth, L. W., and Shaw, J. L., *J. Biol. Chem.*, 1947, **170**, 537.

Does "Animal Protein Factor" Occur in Green Plants?

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A situation of more general interest than was at first anticipated developed in the poultry nutrition field when attempts were made to raise poultry on all-plant rations for the purpose of conserving animal protein sources. The attempts failed and since amino acid supplements did not remedy the flaw, it was postulated that animal proteins have associated with them a factor necessary for chick nutrition. The designation "animal protein factor" came into use. Further work with deficient diets and especially attempts at microbiological assay have raised questions as to its multiplicity.

Very significant progress was made when Bird and associates¹ found that there is a factor in cow manure which has the full effect of the so-called animal protein factor as it is represented by fish meal. From dried cow manure concentrates were made (Bird)² having approximately 1,000 times the potency of the original material. The deficient rations were of the natural ration type (not purified) in which soybean meal was used as a protein source and led to low hatchability and poor growth of the young.

In rats, we have carried out studies³ on all-plant rations, based on either cottonseed meal or soybean meal as a protein source and containing yeast or yeast extract. The deficiency state was best observed in following the growth of offspring from weaning time on. As in the case of the chick studies, mammalian liver, a component of fish (the so-called "fish solubles"), and a 1 to 100 cow manure concentrate relieved the deficiency. Crude casein also contains the missing factor.

Cary and associates⁴ have exhaustively extracted crude casein and thereby removed material essential to growth and well-being of the offspring of rats. Their dams were fed a diet containing such casein and 10% dried yeast together with the known nutritional factors. Data are reported on the growth of the young from 4 weeks on. Among the supplements which led to improvement in growth were milk, cheese, meat, crude casein and liver, as well as dried grasses and alfalfa.

The question arises whether in these 3 types of experiments it is reasonable to consider the deficiency as of the same nature. One obstacle is to be found in the fact that the diet of Bird and associates^{1,2} and that of Zucker and Zucker³ contain 5% of alfalfa and on the diet of Cary and associates alfalfa is an effective supplement.^{4a,b,c} The experiments given below were undertaken to throw some light on this question.

Experimental. Three sources of leafy material have been tried—alfalfa leaf meal, dried young cereal grass (Cerophyll Laboratories) and fresh cut spring lawn grass. The last source was included since in drying grasses may lose some of their nutritional properties. The fresh moist grass was easily made into a homogeneous mixture with the diet by means of a meat grinder. A 2 days' supply was made up each time.

In the first series of experiments, weanling rats from mothers on basal diet Pr 60, previously described by us, were given supple-

¹ Rubin, M., and Bird, H. R., *J. Biol. Chem.*, 1946, **163**, 387, 393.

² Bird, H. R., *Yearbook of Agriculture*, U. S. Dept. Agriculture, 1947, p. 235.

³ Zucker, L. M., and Zucker, T. F., *Arch. Biochem.*, 1948, **16**, 115.

⁴ (a) Cary, C. A., and Hartman, A. M., *Yearbook of Agriculture*, U. S. Dept. Agriculture, 1947, p. 779; (b) Hartman, A. M., and Cary, C. A., U. S. Dept. Agriculture, Bur. of Dairy Ind. BDIM-Inf. 53, Sept., 1947; (c) Hartman, A. M., *Fed. Proc.*, 1946, **5**, 137; (d) Cary, C. A., Hartman, A. M., Dryden, L. P., and Likely, G. D., *Fed. Proc.*, 1946, **5**, 128; (e) Hartman, A. M., Dryden, L. P., and Cary, C. A., *J. Biol. Chem.*, 1941, **140**, liv.

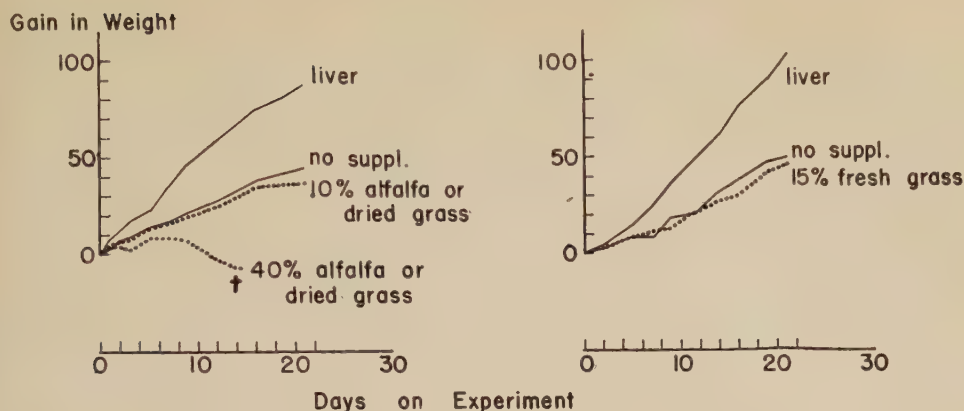


FIG. 1.

The effect of additional leafy feed and of liver supplements on rats raised to weaning on a zoopherin-deficient diet containing 5% alfalfa. There are 4 animals in each experimental group.

ments of 10% and 40% alfalfa leaf meal or dried grass or 15% fresh grass in addition to the 5% alfalfa already in the diet, to determine whether the zoopherin* requirement can be supplied by very high levels of such leafy materials. Fig. 1 compares the growth on the various supplements with the growth on the unsupplemented diet. Controls from the same litters with 5% of 1:20 Wilson liver powder are also shown. The results with alfalfa and with dried grass are averaged because they are indistinguishable. The deaths on the 40% level are perhaps ascribable to the excess of fiber and the resulting low caloric density superimposed on the deficiency. The fact that in spite of dilution of the diet with large amounts of fiber Cary and associates^{4a,b,c} obtained positive results emphasizes the potency of the leaf material under their experimental conditions. The difference in the results on Cary's diet and ours is therefore more striking than appears on the surface.

In a second type of experiment, we have tried the effect of omitting the alfalfa entirely.

* Zoopherin is defined experimentally as the water soluble factor which restores post-lactation growth to normal in the offspring of dams on all-plant diets. The term is used for convenience and to distinguish it from other substances which have been described as "animal protein factor." This paper also inquires into its distinction from unknown growth factors occurring in leafy material which may be stored in the liver or transferred to the young through the milk.

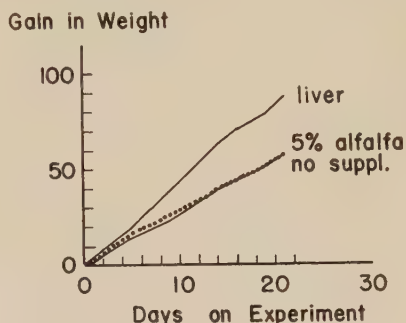


FIG. 2.

The effect of alfalfa and of liver supplements on rats raised to weaning on a zoopherin-deficient diet containing no alfalfa or hay. There are 3 animals per group.

Two stock females were placed at the time of impregnation on basal diet Pr 60, alfalfa being omitted; the young at weaning were divided into 3 groups, one continuing on the same regime, and the other 2 getting supplements of 5% alfalfa leaf meal or 5% 1:20 liver powder. As seen in Fig. 2, alfalfa added at weaning to a diet previously free of it produces no improvement in growth, while liver is a very effective supplement. Neither did the absence of the alfalfa in the diet interfere with good lactation as measured by the 3 weeks' weights of the young (averaging 41 g) or the dams' gain during lactation (averaging 20 g). The observation that zoopherin deficiency itself does not disturb lactation has been described^{4,5} and comparable data under

⁵ Zucker, T. F., and Zucker, L. M., in press.

various conditions have been given.

The results indicate that the alfalfa effect in the experiments of Cary and associates⁴ is not due to the same factor for which our procedure tests. It appears likely that, aside from the principal factor under consideration the yeast and pure vitamin preparations which Cary and associates have in their diet do not satisfy the conditions for best growth of the young. Whatever significant nutritional factors occur in alfalfa are in our case adequately supplied by other diet components, probably the large amount of cottonseed flour.

Discussion. The data constitute evidence on two points: 1) that the amounts of alfalfa, dried grass or fresh grass fed do not contain effective amounts of zoopherin and 2) that whatever unknown factors affecting growth of young rats are contained in alfalfa are also otherwise provided by the all-plant ration. The first of these points stands on its own feet. The second becomes more significant if there is other evidence for a factor in alfalfa which can play the role we attribute to it in the experiments of Cary and associates.⁴ Ball and Barnes⁶ have reported that purified diets containing 8% of yeast are not adequate for lactation of mice (measured by the pre-

weaning growth). Both dehydrated cereal grass and wheat bran effectively improved the situation but these two substances when added to the basal diet alone or combined did not give as good results as the stock commercial diet. Bowland, Ensminger and Cunha⁷ in observations with purified diets (alcohol extracted casein, pure B factors, etc.) on rats have found that when 15% of dried young alfalfa was used as a supplement, 43% of the young given to the female to raise, were weaned. This contrasts with 13% weaned when the same 10 B factors, including folacin were given without alfalfa. Spitzer and Phillips⁸ have also shown evidence for a marked effect of alfalfa on lactation in rats. Their later brief communication,⁹ however, indicates that at least a part of this is attributable to known factors.

Summary. A postweaning growth inhibition in the offspring of rats on a well fortified all-plant ration has been described. While this is corrected by a factor in liver, neither alfalfa, leaf meal, dried grass nor young fresh grass have such an effect.

⁷ Bowland, J. P., Ensminger, M. E., and Cunha, T. J., *Arch. Biochem.*, 1948, **16**, 257.

⁸ Spitzer, R. R., and Phillips, P. H., *J. Nutrition*, 1946, **32**, 631.

⁹ Spitzer, R. R., and Phillips, P. H., *Fed. Proc.*, 1947, **6**, 422.

⁶ Ball, Z. B., and Barnes, R. H., *Proc. Soc. Exp. Biol. and Med.*, 1941, **48**, 692.

16508

Inhibition of Growth of the Vaccinia Virus by β -2-Thienylalanine and its Reversal by Phenylalanine.*†

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The effect of β -2-thienylalanine upon the growth of *Saccharomyces cerevisiae*, *Escherichia coli* and certain other microorganisms has

been reported by du Vigneaud and coworkers.^{1,2} Thienylalanine was observed to inhibit the growth of these organisms, and the inhibi-

* Supported in part by a grant from Burroughs, Wellcome and Company.

† Presented in part at the meeting of the Society of American Bacteriologists at Minneapolis, Minn., in May, 1948.

¹ du Vigneaud, V., McKennis, H., Jr., Simmonds, Sofia, Dittmer, K., and Brown, G. B., *J. Biol. Chem.*, 1945, **159**, 385.

² Dittmer, K., Ellis, G., McKennis, H., Jr., and du Vigneaud, V., *J. Biol. Chem.*, 1946, **164**, 761.

tion was counteracted by phenylalanine. These investigators found that as the concentration of phenylalanine in the medium was increased, larger amounts of thienylalanine were required to produce inhibition of growth.

In a previous report,³ it was demonstrated that the growth of the vaccinia virus in cultures containing chick embryonic tissues was inhibited by a number of substituted amino acids. It was of interest, therefore, to determine whether thienylalanine might act in a similar manner. Experiments also were carried out to ascertain whether phenylalanine or methionine would neutralize the toxicity of thienylalanine for the virus.

Methods. The same strain of vaccinia virus was used for all experiments. Cultures were prepared in tubes with a diameter of 30 mm. The medium consisted of Tyrode's solution containing approximately 1% minced chick embryonic tissue and 2% normal rabbit serum. Supernatant fluid from a previously titrated culture served for the inoculum. The volume of fluid in each culture was 15 ml. Test materials were dissolved in distilled water. Solutions were adjusted to approximately pH 6.5 and filtered through Seitz filters.

Cultures were incubated at 33°C for 96 hours. The final concentration of virus in cultures was determined by the intradermal inoculation of 0.2 ml quantities of decimal dilutions in rabbits. The initial concentration of virus was calculated from data for the culture which supplied the inoculum. Titers were estimated by the method of Reed and Muench.⁴

Results. The results of experiments to determine the effect of thienylalanine, phenylalanine and methionine on the growth of the vaccinia virus are presented in Table I.

Phenylalanine in a concentration of 5×10^{-5} had no demonstrable effect on the multiplication of the virus. On the other hand, the addition of thienylalanine to cultures to give a final concentration of 5×10^{-4} produced a reduction in the concentration of virus. The addition of both compounds to

TABLE I.
Effect of β -2-thienylalanine, Phenylalanine, and Methionine on Growth of Vaccinia Virus in Chick Embryonic Tissues.

Metabolite	Analog	Concentration of		In control cultures	Change in titer of virus (log)		
		Metabolite	Analog		In cultures containing		
					Metabolite	Analog	Metabolite and analog
<i>dl</i> -phenylalanine , , , ,	β -2-thienylalanine	5×10^{-5}	5×10^{-4}	1.85	1.71	-1.02	1.19
	,,	5×10^{-5}	1×10^{-3}	0.90		-2.16	1.14
	,,	2×10^{-5}	1×10^{-3}	,,			0.34
	,,	1×10^{-5}	,,	,,			-0.07
Methionine	,,	1×10^{-4}	,,	1.80	0.62	-2.08	-1.98

³ Thompson, R. L., *J. Immunol.*, 1947, **55**, 345.

⁴ Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, **27**, 493.

cultures permitted viral multiplications.

In a second experiment a constant amount of thienylalanine was added to cultures while the concentration of phenylalanine was varied. The concentration of thienylalanine employed was 1×10^{-3} and the reduction in virus content of cultures was greater than that observed in the first experiment. A ratio of phenylalanine to thienylalanine of 1 to 20 permitted normal growth of the virus. With ratios of 1 to 50 and 1 to 100 a gradual decrease in the neutralization of the toxicity of thienylalanine for the virus was observed.

In view of the similarity in structure of thienylalanine and methionine, tests were made to determine whether the latter compound might counteract the toxicity of the analog for the vaccinia virus. A ratio of methionine to thienylalanine of 1 to 10 failed to permit viral multiplication.

Discussion. The use of metabolite antagonists has greatly added to our knowledge

of the metabolism of bacterial cells.^{5,6} It is reasonable to assume that these compounds likewise can supply valuable information concerning the nutritional requirements of viruses. The observation that thienylalanine inhibits multiplication of the vaccinia virus in chick embryonic tissues and that the toxicity of the analog is relieved by phenylalanine indicates that the metabolism of phenylalanine is in some way associated with the proliferation of the virus. Whether the action of thienylalanine is directed at the virus or at the parasitized cell or both remains to be determined. In any case, thienylalanine is an effective metabolite antagonist in the presence of living tissues.

Summary. β -2-thienylalanine prevents the multiplication of the vaccinia virus in chick embryonic tissues. The toxicity is neutralized by phenylalanine but not by methionine.

The authors are indebted to Dr. Karl Dittmer of the University of Colorado for the thienylalanine used in these experiments.

⁵ Roblin, R. O., Jr., *Chem. Rev.*, 1946, **38**, 255.

⁶ Woolley, D. W., *Physiol. Rev.*, 1947, **27**, 308.

16509

Growth of Neurotropic Viruses in Extraneural Tissues. I. MM Virus in the Feet of Hamsters.

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Evidence from many sources suggests that the viruses of poliomyelitis and mouse encephalomyelitis grow in extraneural tissues.¹ It is well known that these viruses occur frequently in the intestinal contents and feces of infected persons and laboratory animals. Numerous reports of finding them in extraneural tissues outside of the intestinal tract of infected persons and monkeys²⁻¹⁸ and of rodents¹⁹⁻²⁶ have been published. There have been several reports of the growth of some strains in tissue culture^{21,27,28} and in the chick embryo.^{29,30,31}

A precise identification of the extraneural tissues capable of supporting growth of these viruses in the intact animal has not been re-

² Burnet, F. M., and Jackson, A. V., *Australian J. Exp. Biol. and Med. Sci.*, 1940, **18**, 361.

³ Flexner, S., and Amoss, H. L., *J. Exp. Med.*, 1919, **29**, 379.

⁴ Flexner, S., and Lewis, P. A., *J. A. M. A.*, 1910, **54**, 535.

⁵ Horstmann, Dorothy M., Melnick, J. L., and Wenner, Herbert A., *J. Clin. Invest.*, 1946, **25**, 270.

⁶ Horstmann, Dorothy M., Melnick, Joseph L., Ward, Robert, and Sá Fleitas, M. José, *J. Exp. Med.*, 1947, **86**, 309.

¹ Evans, C. A., and Green, R. G., *J. A. M. A.*, 1947, **134**, 1154.

ported in the case of any one strain of virus or any one species of animal.

The relationship between the various strains of viruses designated as poliomyelitis and mouse encephalomyelitis is obscure. They

⁷ Howe, H. A., Wenner, H. A., Bodian, D., and Maxcy, K. F., *PROC. SOC. EXP. BIOL. AND MED.*, 1944, **56**, 171.

⁸ Howe, Howard A., and Bodian, David, *Am. J. Hygiene*, 1947, **45**, 219.

⁹ Howe, Howard A., Bodian, David, and Wenner, Herbert A., *Bull. Johns Hopkins Hosp.*, 1945, **76**, 19.

¹⁰ Kessel, J. F., Moore, F. J., Stimpert, F. D., and Fisk, R. T., *J. Exp. Med.*, 1941, **74**, 601.

¹¹ Kling, C., Olin, G., and Gard, S., *C. R. Soc. de Biol.*, 1938, **129**, 451.

¹² Melnick, Joseph L., *PROC. SOC. EXP. BIOL. AND MED.*, 1945, **58**, 14.

¹³ Melnick, Joseph L., Horstmann, Dorothy M., and Ward, Robert, *J. Clin. Invest.*, 1946, **25**, 275.

¹⁴ Paul, John R., Trask, James D., and Webster, Leslie T., *J. Exp. Med.*, 1935, **62**, 245.

¹⁵ Sabin, A. B., and Ward, R., *J. Exp. Med.*, 1941, **73**, 771.

¹⁶ Ward, R., and Waters, B., *Bull. Johns Hopkins Hosp.*, 1947, **80**, 98.

¹⁷ Ward, R., Horstmann, D. M., and Melnick, J. L., *J. Clin. Invest.*, 1946, **25**, 284.

¹⁸ Wenner, H. A., and Paul, J. R., *Am. J. Med. Sci.*, 1947, **213**, 9.

¹⁹ Gard, S., *Yale J. Biol. and Med.*, 1944, **16**, 467.

²⁰ Jungeblut, C. W., and Sanders, M., *J. Exp. Med.*, 1940, **72**, 407.

²¹ Jungeblut, C. W., Feiner, R. R., and Sanders, M., *J. Exp. Med.*, 1942, **76**, 31.

²² Olitsky, P. K., *J. Exp. Med.*, 1940, **72**, 113.

²³ Sanz Ibanez, J., *Trab. d. Inst. Cajal de Invest. Biol.*, 1944, **36**, 137.

²⁴ Smith, M. G., *PROC. SOC. EXP. BIOL. AND MED.*, 1943, **52**, 88.

²⁵ Theiler, M., and Gard, S., *J. Exp. Med.*, 1940, **72**, 49.

²⁶ Theiler, M., *Med.*, 1941, **20**, 443.

²⁷ Parker, Raymond C., and Hollender, Augusta J., *PROC. SOC. EXP. BIOL. AND MED.*, 1945, **60**, 88.

²⁸ Sanders, M., and Jungeblut, C. W., *J. Exp. Med.*, 1942, **75**, 631.

²⁹ Brutsaert, Paul, Jungeblut, Claus W., and Knox, Alice, *J. Pediatrics*, 1946, **29**, 350.

³⁰ Gard, S., *Nature*, 1943, **152**, 660.

³¹ Schultz, Edwin W., and Enright, John B., *PROC. SOC. EXP. BIOL. AND MED.*, 1946, **63**, 8.

clearly differ greatly in pathogenicity for different kinds of animals but there is at present no satisfactory means of classifying them. A knowledge of the ability of representative strains to grow in different kinds of tissue cells should be of value in developing a reasonable system of classification.

In the experiments reported in this paper, one member of this group, the MM virus, was shown to increase rapidly and extensively in the inoculated foot of the Syrian hamster.

The MM virus was originally isolated and described by Jungeblut and Dalldorf.³² The virus used in these investigations was obtained from Dr. Gilbert Dalldorf in January 1944, and subsequently has been maintained by a total of less than 20 passages in mouse brains with intervals of storage as infected mouse brain in 50% neutral glycerin at 4°C. During the time the experiments were in progress it was shown to be noninfective for guinea pigs by subcutaneous inoculation and for rabbits by intra-ocular inoculation. Mice injected intracranially with the virus died at 2 or 3 days, or with minimal infective doses, after periods up to 7 days. Occasional mice exhibited extensive paralysis before death.

The hamsters used in these experiments were raised in the departmental animal colony and were 4 to 5 weeks old. Hamsters injected subcutaneously with MM virus regularly developed paralysis. There was no definite pattern to the areas of involvement, facial muscles, as well as limbs being paralyzed in various animals. A hamster inoculated in the right hind foot after excision of a section of the right sciatic nerve developed extensive paralysis after the usual incubation period of 3 days.

Procedure. A stock supply of virus was prepared as a 20% suspension of infected mouse brain in 50% neutral glycerine. This was kept in a vaccine bottle at 4°C. Titrations showed that this stock suspension maintained its potency well throughout the period of the experiments.

Hamsters were injected subcutaneously in the pad of the right hind foot with 0.03 ml of

³² Jungeblut, C. W., and Dalldorf, G., *Am. J. Pub. Health*, 1943, **33**, 169.

TABLE I.
Tests for Virus in the Blood and Central Nervous System of Hamsters Inoculated with MM
Virus in the Hind Foot.
Exp. I. Sept. 1, '47.

Hr. after injection	12*	36*	61†
Dilution of tissue	10-1	10-1	10-1
Blood	4	2	2
	S	3	3
	S	3	5
CNS	10	2	2
	S	3	2
	S	3	3

* Pool from 3 hamsters.

† Pool from 2 hamsters.

Hamsters in this experiment received 0.03 ml 10-3 dilution of MM virus.

Exp. II. Sept. 8, '47.

Hr after inj.	8	24			36			48		
Dilution of tissue	10-1	10-1	10-2	10-3	10-1	10-2	10-3	10-1	10-2	10-3
Blood*	4	3	2	3	3	2	3	2	2	2
	S	3	3	4	3	3	4	2	2	6
	S	3	3	S	3	4	6	2	2	S
CNS*	S	3	S	S	2	3	3	1	2	2
	S	4	S	S	3	3	3	2	2	2
	S	S	S	S	3	3	3	2	3	2

* Pool from 2 hamsters.

Hamsters in this experiment received 0.03 ml 10-4 dilution of MM virus.

inoculum. Subsequently various tissues were tested quantitatively for the amount of virus present.

Dilutions of virus, or of tissues being tested for virus, were prepared in Ringer's solution containing glucose. Tests for virus were all based on the intracranial injection of mice approximately 3 to 4 weeks of age.

Experimental. I. Determination of the approximate minimal infective dose (m.i.d.) for hamsters inoculated in the hind foot.

In a preliminary experiment it was determined that a 10⁻⁵ dilution of infected mouse brain (from the stock suspension) constituted approximately 1 m.i.d. of virus for hamsters inoculated in the foot pad. In subsequent experiments, hamsters were injected with a 10⁻⁴ dilution (10⁻³ dilution in one experiment) of infected brain from the same stock suspension.

II. Virus increases in the blood before it is detectable in the central nervous system (CNS).

In a series of several experiments (Table I) it was shown that after MM virus is inocu-

lated subcutaneously into the pad of a hind foot of a hamster, a definite increase in the amount of virus in the blood precedes the appearance of and growth of virus in the central nervous system. In one experiment, for example, 28 hours after injection of virus into the foot pad, tests showed that 2 out of 3 mice injected with blood diluted to 10⁻² were infected, but a 10% suspension of brain and spinal cord from the same hamster failed to infect mice.

The results strongly suggested that the virus was growing in the extraneural tissues. We next sought to determine the site of this growth of virus.

III. Virus increases in tissue at the site of injection before appearing in the blood.

Eight hamsters 34 days old from the same litter were inoculated in the right hind foot with a 10⁻⁴ dilution of infected mouse brain. Two animals were killed at 20, 24, 28 and 32 hours.

The initial injections were made at 9 A.M. (animals killed 24, 28, and 32 hours), and 1 P.M. (hamsters killed at 20 hours) on the

Concentration of MM Virus in Tissues of Hamsters Killed at Intervals After Inoculation of Virus into the Hind Foot

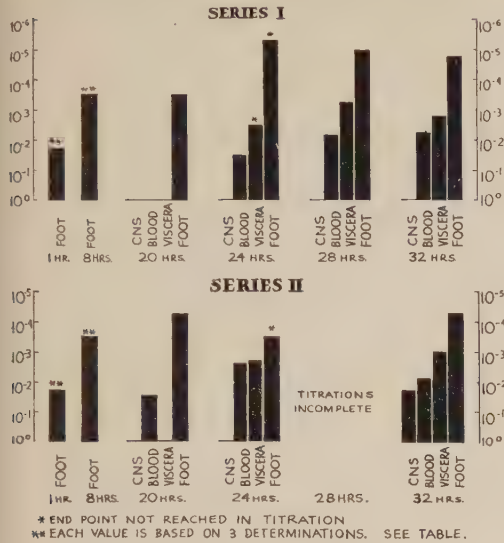


FIG. 1.

Concentration of MM Virus in Tissues of Hamsters Killed at Intervals After Inoculation of Virus into the Hind Foot.

Results of tests on individual hamsters killed at 20, 24, 28, and 32 hours are shown. Data for the 1- and 8-hour periods represent the average titers of 3 tests at each time period and are duplicated under Series I and Series II.

"Foot" designates tissue at the site of inoculation in the hind foot.

Note the early increase of virus in the foot with a subsequent rise in virus of the blood and viscera before it was present in the CNS.

same day. Titrations of virus in the inoculum showed that in spite of the fact that it was kept in the refrigerator from 9 A.M. to 5 P.M., a significant amount of virus died during this interval. Fortunately, the critical results were obtained with hamsters inoculated at 9 A.M.

Hamsters were killed with ether at the appropriate times on the following day. A sample of blood was collected from the heart. The brain and cervical spinal cord (CNS) were removed and tested for virus and the carcass placed in a freezer at -40°C .

At intervals of 3 to 19 weeks following the killing of the animals and the initial tests of blood and CNS, frozen carcasses were removed from the freezer and studied more extensively. There was no indication that appreciable

amounts of virus died during storage. Tissues tested included local tissue from the site of injection of the foot, right popliteal lymph node, liver, kidney, spleen, and blood. The local tissue from the foot pad was obtained by reflecting the skin of the foot and dissecting off the remaining soft tissue of the plantar side of the foot. This was carefully weighed and amounted to from 16 to 31 mg. No inflammation was evident. The popliteal lymph nodes were found without difficulty and were determined to weigh from 2.2 to 7.0 mg. Portions of liver, spleen and kidney weighing approximately 0.1 to 0.5 g were tested individually or as pooled suspensions. In preparing the pooled suspensions, the portions of liver, spleen and kidney from the same animal were pooled in a sterile mortar, triturated thoroughly, and Ringer's solution added to make a 10% suspension.

Initial suspensions of abdominal viscera and CNS were 10^{-1} . In the case of local tissue and lymph nodes, 10^{-2} suspensions were the most concentrated that could be injected.

The results of this experiment are shown in Table II, and are presented graphically in Fig. 1. All end points were calculated by the method of Reed and Muench.³³ All deaths over 7 days were omitted in these calculations but are included in the tables.

It is clearly shown that within 32 hours after inoculation, virus attained a very high concentration in the tissues of the inoculated foot. In animals killed at the proper stage of the infection, there is a gradient of decreasing virus content from the foot (which contained the largest amount) to the regional lymph node, the blood and viscera, and central nervous system. In a single test the tissues of the uninoculated left hind foot of a hamster did not contain a detectable amount of virus at 32 hours, although there was sufficient virus in the inoculated right hind foot at this time to kill 3 out of 3 mice injected with a 10^{-4} dilution and 1 out of 3 injected with a 10^{-5} dilution of the tissue.

In subsequent experiments the inoculated

³³ Reed, L. J., and Muench, H., *Am. J. Hygiene*, 1938, **27**, 493.

Dilution	Hamster II, 20 hr					Hamster II, 24 hr					Hamster II, 28 hr					Hamster II, 32 hr					
	10-1	10-2	10-3	10-4	10-5	10-1	10-2	10-3			10-1	10-2	10-3			10-1	10-2	10-3	10-4	10-5	10-6
CNS	2	2				2	2				4	2				3	3				
	2	2				2	2				2	2				4	2				
	2	2				2	2				2	2				4	2				
Local tissue		2	2	3	2		2	2			2	2	2			2	2	2	3	2	2
		2	2	4	2		2	2			2	2	3			2	2	3	5	2	2
		2	3	2	2		2	3			3	3	3			2	2	3	2	2	2
Lymph node		2					2	2			2	2				2	2	1	3	2	2
		2					2	3			2	2				3	3	3	2	2	2
		2					2	3			3	3				4	4	3	2	2	2
Blood	3	4				2,3*	3,2*	5*			2	2				2,3*	3,3*	2*	2*		
	3	2				2,3	4,4	2			2	3				2,3	3,9	2	2		
	2	2				2,3	2,5	2			3	4				3,2	3,2	2	2		
Viscera	2	2														2	3	3	2	2	2
	2	2														3	4	5	2	2	2
	2	2														3	13	2	2	2	2
Spleen																					
Liver																					
Kidneys																					

CNS and blood of all hamsters were tested 9/16. Other tissues were tested on the following dates: Hamster I, 20 hr, 10/20; Hamster II, 20 hr, 1/26; Hamster I, 24 hr, 11/3; Hamster II, 24 hr, 10/13; Hamster I, 28 hr, 11/29; Hamster II, 28 hr, 10/6; Hamster I, 32 hr, 11/17; Hamster II, 32 hr, 11/28.

A second test of blood of the following hamsters was made at the time other tissues were tested: Hamster II, 24 hr, Hamster I, 28 hr, Hamsters I and II, 32 hr.

TABLE III.
Tests for Amounts of Virus in Local Tissue of Hind Feet of Hamsters at Intervals of 1, 7, and 8 Hr After Injection of MM Virus.
Tests at 7 hr. (Exp. I. Injections Feb. 2, feet frozen over night before tests for virus.)

Dilution of tissue	Hamster I.		Hamster II.	
	10-2	10-3	10-2	10-3
	2	6	3	3
	3	S	3	6
	4	S	S	S

Date of test*	Tests at 1 hr. (Exp. II. Injections Mar. 12). Hamster I. Right hind foot Mar. 20, '48		Hamster I. Left hind foot Mar. 23, '48		Hamster II. Right hind foot Mar. 15, '48	
	10-2	10-3	10-2	10-3	10-2	10-3
Dilution of tissue	3	S	3	S	4	S
	S	S	S	S	S	S
	S	S	S	S	S	S

Date of test*	Tests at 8 hr. (Exp. II. Injections Mar. 12.) Hamster II. Left hind foot Mar. 15, '48			Hamster III. Right hind foot Mar. 20, '48			Hamster III. Left hind foot Mar. 23, '48		
	10-2	10-3	10-4	10-2	10-3	10-4	10-2	10-3	10-4
Dilution of tissue	2	3	S	2	2	S	3	3	6
	2	4	S	3	3	S	3	4	S
	3	S	S	3	4	S	3	5	S

* Feet frozen until time of test.

feet of hamsters were tested at 1, 7 and 8 hours after injection of a 10^{-4} suspension of MM virus. The feet were frozen for 1 to 11 days before tests for virus were made. The results, as shown in Table III and in Fig. 1, indicate that even as early as 7 to 8 hours after injection there was considerable growth of virus in the inoculated feet.

Summary and conclusion. After inoculation

into the pad of the hind foot of a hamster, MM virus increases in amount in the local tissue. Subsequent increases in virus in the blood and viscera are in turn followed by appearance of the virus in the central nervous system. It appears certain that the virus grows in the foot. Whether the growth occurs in subcutaneous tissue, muscles, or nerve endings was not determined in these experiments.

16510

The Mechanism of Egg-White Inhibition of Hemagglutination by Swine Influenza Virus.*

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Egg-white (EW) has been found capable of inhibiting hemagglutination by purified

preparations of swine influenza virus and derivatives obtained by heating the virus or treating it with formaldehyde.¹ This capacity

* This work was aided by a grant to Duke University from the Lederle Laboratories, Inc., Pearl River, N.Y.

¹ Lanni, F., and Beard, J. W., *PROC. SOC. EXP. BIOL. AND MED.*, 1948, **68**, 312.

of EW showed an interesting dependence on the history of the virus preparation. Thus, the EW inhibition titers for heated virus and for an aged formolized swine influenza vaccine averaged approximately 170,000 and 54,000, respectively, while the titers for highly infectious, untreated preparations, which we have designated SF, may be as low as 50 to 100.

Such a dependence of the inhibition titer on the character of the virus preparation was first described by Francis,² who found that the ordinarily low inhibition titer of normal serum for influenza virus B hemagglutination was greater by a factor up to 128, but more typically 32 to 64, if the virus preparation had been heated at 56°C for 30 minutes. This behavior, which has been observed also with other body fluids and tissue extracts and has recently been the subject of several extensive investigations,^{3,4} stands in marked contrast with the considerably greater effects observed with EW.

In a previous report,¹ evidence has been presented that the mechanism of EW inhibition of hemagglutination by swine influenza virus involves combination between an EW component and virus. Further evidence on this point, as well as on the great disparity in inhibition titers for the active, untreated swine influenza virus and those for the vaccine, is provided in the present communication.

Materials and methods. The swine influenza virus was the egg-adapted strain which has been employed in other studies in this laboratory⁵ and which came originally from Dr. R. E. Shope; it has been designated as Strain 15. The active, untreated virus (SF) used in the present work was obtained from a pool of chorio-allantoic fluid from 4,269 eggs infected with the agent and harvested as previously described.⁶ Concentration of the virus was effected by spinning 27.8 l of the fluid

in the modified Sharples centrifuge⁶ at 47,000 g at a flow rate of 1.5 l per hour. The virus concentrate in a volume of 220 ml was spun in the angle centrifuge at 1,600 g for 10 minutes. The virus in a 90-ml portion of the supernatant fluid was then sedimented in the vacuum type centrifuge by spinning at 20,000 g for 1 hour. The large pellets, showing little opaque material, were dispersed in 240 ml of Ringer solution and centrifuged in the angle head at 1,600 g for 10 minutes. These manipulations were completed on 11/6/47 and the virus, stored at 0.234 mg N per ml at about 4°C, had a 50% endpoint infectious unit of $10^{-14.7}$ g N on 2/9/48.

The vaccine was a Sharples concentrate of swine influenza virus obtained as previously described⁷ and treated with 0.05% formalin and 1:50,000 phenyl mercuric borate on 5/22/44. It had been stored at about 4°C in the interim.

Hemagglutinative capacity was determined by the method of Hirst,⁸ and the test mixtures were compared visually with the standard red blood cell suspensions.⁵ The diluent throughout was buffered saline which consisted of a solution of 0.81% sodium chloride and 0.005 M phosphate at pH 7.3. The inhibition titer is defined as the reciprocal of the dilution at which the standard 2-plus endpoint, signifying the inhibition of all but 1 hemagglutinating dose (HD), was obtained. Generally, 4 HD were added, and 3 HD were inhibited. Variations arose from experiment to experiment because of the difficulty in determining the hemagglutinating dose precisely.

The egg-white was separated from the yolk and forced through several layers of fine-meshed gauze supported on a wire screen in a 20-ml syringe. Further mixing of the white before sampling was accomplished by repeated pipetting.

Experimental. An experiment was carried out to determine the dependence of the inhibition titer on (a) the order in which the reagents EW, vaccine and chicken RBC were

² Francis, T., Jr., *J. Exp. Med.*, 1947, **85**, 1.

³ Burnet, F. M., *Lancet*, 1948, **254**, 7.

⁴ Hirst, G. K., *J. Exp. Med.*, 1948, **87**, 315.

⁵ Taylor, A. R., Sharp, D. G., McLean, I. W., Jr., Beard, D., Beard, J. W., Dingle, J. H., and Feller, A. E., *J. Immunol.*, 1944, **48**, 361.

⁶ Taylor, A. R., Sharp, D. G., McLean, I. W., Jr., Beard, D., and Beard, J. W., *J. Immunol.*, 1945, **50**, 291.

⁷ McLean, I. W., Jr., Beard, D., Taylor, A. R., Sharp, D. G., and Beard, J. W., *J. Immunol.*, 1945, **51**, 65.

⁸ Hirst, G. K., *J. Exp. Med.*, 1942, **75**, 49.

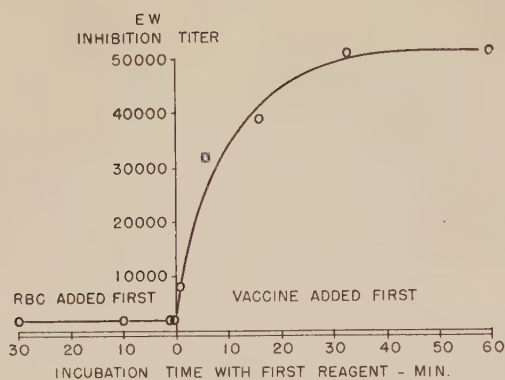


FIG. 1.

Inhibition titer as a function of the order of addition of chicken red blood cells and vaccine to egg-white (EW) dilutions and the time of incubation at 28°C with the reagent added first. The ordinate of each point denotes the reciprocal of the final dilution of EW at which the standard 2-plus agglutination endpoint was obtained in a titration involving constant RBC, constant vaccine and a set of final EW dilutions ranging from 1:500 to 1:512,000. The only variables among the titrations were the order of addition of RBC and vaccine to the EW dilutions and the elapsed time between the addition of these two reagents.

mixed, and (b) the time of incubation of EW with one of the reagents, vaccine or RBC, before the other reagent was added. Progressive 2-fold dilutions of EW were made with buffered saline, and 0.5-ml portions of each dilution were distributed in several tubes so as to provide several sets of dilutions. To each tube of some of the sets was added 1.0 ml of 2% RBC, and to each tube of other sets 0.5 ml of a vaccine dilution containing 8 HD per ml. After different periods of incubation at room temperature (28°C), the appropriate amount of the second reagent, RBC or vaccine, was added to bring all the sets to the same reagent composition. After an additional hour, the degree of agglutination was determined in the usual manner. Fig. 1, which summarizes the results, shows that the EW inhibition titer was experimentally invariant, at the level 2,000, with respect to the time of incubation of EW with RBC prior to the addition of vaccine. On the other hand, with extended incubation of EW with vaccine before the addition of RBC, the titer showed a progressive rise, attaining a maximum of 51,000 after about 30 minutes incubation. Moreover, when a fresh mixture

of RBC and vaccine was quickly added to EW dilutions, the inhibition titer was only 500. These results provide strong evidence that EW is effective as inhibitor through obstructive combination with vaccine, and not with RBC. Accordingly, we may explain the several inhibition titers observed in this experiment in terms of the outcome of a mutually exclusive competition of EW and RBC for combining groups of the vaccine, a particular titer depending on which of these reagents is given the greater opportunity of reacting with vaccine.

A similar experiment in which EW dilutions were incubated at 26°C with 4 HD of active, untreated swine influenza virus (SF) for varying periods prior to the addition of RBC gave, however, a quite different result, the inhibition titer *decreasing* from a value of 80 after one minute of incubation to a value of 20 after about 15 minutes. At 22°C, a titer of 320 after one minute of incubation was followed by a titer of 160 after 15 minutes and a titer of 80 after one hour. This rapid decrease in titer, manifested by progressive emergence of the hemagglutinating activity of SF, indicated a capacity of SF to destroy or inactivate, but not merely to neutralize, the EW inhibitor (cf. ^{3,4,9-11}) and provided an explanation for the variation in titers encountered in preliminary experiments with SF when the time of incubation was not under control.

Evidence that the inhibitor effective against vaccine is likewise destroyed by SF and, accordingly, that the same EW component is responsible for inhibition of both SF and vaccine is provided in Table I. Several identical mixtures containing EW in a final concentration of 1:50 and 12 HD of SF per ml were incubated for varying periods at room temperature (25°C) and then diluted 2-fold serially to provide 2 parallel sets of dilutions, with 0.5 ml in each tube. To each tube of one set was added 0.5 ml of vaccine

⁹ Friedewald, W. F., Miller, E. S., and Whatley, L. R., *J. Exp. Med.*, 1947, **86**, 65.

¹⁰ de Burgh, P. M., Yu, P. C., Howe, C., and Bovernick, M., *J. Exp. Med.*, 1948, **87**, 1.

¹¹ Hirst, G. K., *J. Exp. Med.*, 1948, **87**, 301.

TABLE I. Effect of Incubation of Egg-White (EW) with Untreated Swine Influenza Virus (SF) on the Egg-White Inhibitor for SF and for Vaccine.

Min. incubation before dilution	Titrated with	Reciprocal of final dilution of incubated mixture									
		8	16	32	64	128	256	512	1024	2048	
Approx. 1	4 HD* vaccine	++	++	0	0	±	+	++±	++±	++±	
	Saline	++	±	0	0	0	0	0	0	0	
35	4 HD vaccine	++	++	++±	++	++	++	++±	++	++	
	Saline	++±	++±	±	0	0	0	0	0	0	
64	4 HD vaccine	++	++	++	++±	++	++	++±	++	++	
	Saline	++	++	+	0	0	0	0	0	0	
125	4 HD vaccine	++	++	++	++	++	++	++	++	++	
	Saline	++±	++±	±	0	0	0	0	0	0	
EW control (no SF)	4 HD vaccine	0	0	0	0	0	+	++	++	++	
	Saline	0	0	0	0	0	0	0	0	0	
SF control (no EW)	Saline	++	++	++	±	0	0	0	0	0	

* Hemagglutinating doses.

(4 HD), and to each tube of the second set 0.5 ml of saline. After 30 to 40 minutes incubation, to allow maximal reaction of inhibitor with vaccine, 1.0 ml of 2% RBC was added, and the extent of agglutination was determined after one hour. The results (Table I) show progressive destruction of inhibitor for vaccine accompanied by progressive emergence of the hemagglutinating activity of SF. This result suggests, at the same time, the usefulness of vaccine for studying the inactivation of EW inhibitor by SF. In a comparable experiment with vaccine substituted for SF, a slight inactivation of the inhibitor was evident after 5 hours and 19 hours, but the effect was too small to be regarded as significant in the present state of the experiments.

Since the degree of purity of the ultra-centrifugally concentrated virus preparation SF was not known, it was desired to obtain evidence on the nature of the SF constituent which was responsible for the inactivation of EW inhibitor. For this, we resorted to the use of convalescent (anti-influenza) swine serum. Such a serum, obtained from an animal after experimental intranasal infection with active virus in chorio-allantoic fluid,¹² may be regarded as the most specific reagent for this virus at present available, since the opportunity is minimized for the occurrence in it of antibodies directed against materials extraneous to the virus itself. Orienting experiments showed that this serum was capable of neutralizing the hemagglutinative activity of 3 HD of either SF or vaccine in a dilution of 1:2500. A normal swine serum, selected for a control, was approximately 50 to 100 times weaker in this respect.

A basic experiment involving the incubation of EW, in a dilution of 1:50, with the minimal quantity of SF (about 6 to 8 HD per ml of mixture) which caused inactivation of at least 90% of the inhibitor in one hour at room temperature, as determined with vaccine (Table I), was modified slightly to allow the study of the effect of convalescent serum. Mixtures of equal volumes of 1:200

¹² McLean, I. W., Jr., Beard, D., and Beard, J. W., *J. Immunol.*, 1947, **56**, 109.

TABLE II.
Effect of Convalescent (Anti-Influenza) Swine Serum on the Inactivation of Egg-White (EW) Inhibitor by Untreated Swine Influenza Virus (SF)

Initial mixture	After 10 min., room temp., add	After one more hr, add	Reciprocal of final dilution of mixture in titrations with 4 HD* vaccine									
			8	16	32	64	128	256	512	1024	2048	
Saline (2 vols.)	EW	Saline	0	0	0	0	+	+	+	+	+	
Immune serum + saline	Saline	"	+	+	+	+	+	+	+	+	+	
Immune serum + SF	EW	"	0	0	0	0	0	+	+	+	+	
Saline + SF	EW	Immune serum	+	+	+	+	+	+	+	+	+	
Normal serum + SF	EW	Saline	+	+	+	+	+	+	+	+	+	

* Hemagglutinating doses.

serum, 3:50 EW, 22 HD of SF per ml, and saline were prepared as indicated in Table II. After incubation was complete, the mixtures were diluted progressively with saline, and 0.5 ml of each dilution was incubated with 0.5 ml (4 HD) of vaccine for 30 to 40 minutes at room temperature before the addition of RBC. It was found (Table II) that the convalescent serum, added in the amount required to neutralize the hemagglutinative activity of the SF employed, was completely effective in suppressing the inhibitor-inactivating capacity of SF when the serum and SF were incubated together for ten minutes before the addition of EW. On the other hand, normal pig serum, tested in quantities up to 40 times the effective quantity of immune serum, was without demonstrable effect in suppressing inhibitor inactivation; and immune serum added to a previously incubated mixture of SF and EW was likewise ineffective. Accordingly, the suppression of inhibitor inactivation by immune serum may reasonably be ascribed to a combination between the specific antibody and the virus, and, in consequence, the capacity of SF to inactivate EW inhibitor must be ascribed to the virus particles themselves.

Discussion. The evidence from different kinds of experiments clearly indicates that the capacity of egg-white (EW) to inhibit hemagglutination by swine influenza virus rests on the capacity of an unidentified EW component to combine with virus and to obstruct in this way the agglutinative reaction of virus with RBC.

In the course of these experiments, it was found that the EW inhibitor was rapidly inactivated by small quantities of highly infectious virus (SF) but not by the non-infectious virus of an aged formolized vaccine. As EW was incubated with SF, the hemagglutinating activity of SF, initially depressed by EW, gradually emerged until it attained, within experimental error, its full, uninhibited level; and, at the same time, the amount of inhibitor titratable with vaccine was reduced.

To account for this inactivation of EW inhibitor by SF, one may advance two reasonable general hypotheses, an *enzymatic* hy-

pothesis which postulates a capacity of the virus particle, or, more specifically, of a given reactive patch of the virus surface, to inactivate successive molecules of inhibitor, inactivating one first, being released, inactivating a second, and so on; and a *stoichiometric* hypothesis which postulates an upper limit to the amount of inhibitor that can be inactivated by a single reactive patch of the virus, the limit being fixed by the number of inhibitor molecules that can attach *at one time* to the same reactive patch. For the moment we may neglect, since there is no evidence for it, the possibility of penetration of the virus surface by inhibitor molecules, and we may suppose that all of the phenomena must be accounted for in terms of reactions occurring at the surface of the virus particle.

The crucial observations that have to be explained by these hypotheses are (a) the inactivation of a large amount of inhibitor by a small amount of virus, and (b) the progressive emergence of the hemagglutinating activity of virus following an initial period of inhibition. Until the inhibitor has been isolated and weighed, its inactivation by virus must be discussed in terms of inhibitor activity. A calculation shows that one hemagglutinating dose of SF, corresponding to about $10^{-7.6}$ g of virus N (cf.⁵), is capable of inactivating in one hour at room temperature under the conditions of our experiments an amount of inhibitor which is able to suppress the hemagglutinating activity of approximately 100 hemagglutinating doses of vaccine. Since SF and vaccine have, within experimental error, the same hemagglutinating activity per unit weight of N, this is equivalent to saying that a given weight of SF is able to inactivate an amount of inhibitor effective against approximately 100 times that weight of vaccine.

To account for this disproportion by a stoichiometric hypothesis, one is led to suppose that SF is approximately 100 times as effective as vaccine in binding EW inhibitor. Then, to account for the emergence of SF from an initial inhibition, one must further suppose that the reactive patches of the

virus, in stable combination with inhibitor, are dissociated from the rest of the virus particle and that the released minus-patch virus particle retains, or acquires anew, essentially full hemagglutinative activity. It is evident that the stoichiometric hypothesis is so complicated as to make its acceptance undesirable at the present time.

On the other hand, an enzymatic hypothesis offers a relatively simple explanation of the observed phenomena. According to such an hypothesis, the initial reaction of combination of virus with EW inhibitor is followed by destruction of the inhibitor and release of the unmodified virus, enabling it to combine with and destroy yet another molecule of inhibitor. As a consequence, the EW inhibition titer is low. This is true if the virus is of the highly infectious sort such as occurs in SF. If, however, the virus has been modified so that, while retaining its capacity to combine with RBC, specific antibody, and EW inhibitor, it has lost its capacity to destroy the EW inhibitor, the combination of such a modified virus with inhibitor is stable, and the EW inhibition titer is high. Thus, to account for the difference in inhibitor-inactivating capacities of SF and vaccine, one need postulate only a change in the inhibitor-binding patches of the virus, such that the loss of enzymatic capacity is not accompanied by the loss of combining capacity. The great disparity in EW inhibition titers for SF and those for vaccine may be attributed in part to a disparity in the inhibitor-inactivating capacities of SF and vaccine and in part to a second factor which is suggested by the experiment with vaccine recorded in Fig. 1. In this experiment it was found that the EW inhibition titer was greatly dependent on the order of mixing the three reagents vaccine, EW and RBC; in particular, the low titer, 2,000, was obtained when the 3 reagents were mixed simultaneously, in contrast with the high titer, 51,000, obtained when RBC were added to suitably incubated mixtures of EW and vaccine. On the assumption that the competition of EW and RBC for SF is similar to the competition of these reagents for vaccine and that the de-

struction of inhibitor by SF continues during the period of the titrations when RBC are present, we may suppose that, as virus is regenerated from complexes with inhibitor, a system prepared by adding RBC to a mixture of SF and EW progresses toward a system in which the 3 reagents have been mixed simultaneously; consequently, the inhibition titer will be lower than one might predict from a consideration of the amount of inhibitor destruction alone.

It will be seen that this interpretation of the relations among SF, vaccine, and EW inhibitor is in general accord with the explanation recently offered by Burnet³ and by Hirst⁴ for an analogous set of relations involving other viruses and normal serum as inhibitor.

Summary. Evidence has been presented that the mechanism of egg-white inhibition of hemagglutination of chicken red blood cells by swine influenza virus involves combination between an egg-white component and the virus, this combination obstructing the reaction of virus with red blood cells. Moreover, it has been found that the inhibitor is rapidly inactivated by highly infectious virus, with regeneration of the virus, but not by an aged formalized vaccine consisting of inactivated virus; and that the inactivation of inhibitor by virus is suppressed by convalescent (anti-influenza) swine serum. The results have been interpreted in terms of an enzymatic hypothesis of virus function in the phenomenon.

16511

Use of Formalin-Treated Red Cells for the Study of Influenza A Virus Hemagglutinating Activity.

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Human erythrocytes, treated with formalin, the writer has found, retain their ability to react with influenza A virus in a manner quite similar to that of untreated human erythrocytes. For certain types of experiments the use of such formalized red cells possesses distinct advantages over the use of normal red cells.

To prepare formalized red cells, equal volumes of a 50% (by volume) suspension of washed red cells in saline and formalin containing sodium chloride in a concentration of 0.85%, were mixed and allowed to stand for 2 to 3 days. If the formalin concentration of the mixture was between 10 and 50%, the cells became dark brown gradually over a period of 24 hours or so. The cells also became cohesive and very resistant to hemolytic agents. The stability of the cells thus treated was marked. Samples of formalized cells stored at 4°C have retained their ac-

tivity undiminished for influenza A virus for almost two years now. Furthermore, the cells have retained the microscopic appearance and shape of fresh, normal, red cells.

Concentrations of formalin of less than 10% (3.6% formaldehyde) in the mixture failed to produce browning of the red cells over 2-3 days at 4°C usually, but did render the cells cohesive. Such cells hemolyzed more readily in hypotonic solutions than did the cells prepared with higher concentrations of formalin.

Before using the formalized red cells in reactions with influenza virus the uncombined formaldehyde was removed by repeated washing with saline or water. The cells were allowed to stand in contact with each wash fluid for a period of at least several hours to permit the establishment of an equilibrium between the free formaldehyde inside and outside the cells. Generally, 6 to 10 such

washings with about 5 volumes of diluent each, sufficed to yield cells with no obvious odor of formaldehyde and which released such small quantities of formaldehyde into the final wash that testing with Schiff's aldehyde reagent produced only a faint, delayed color reaction.

For studying electrolyte-free systems¹ water has an advantage over saline as the wash fluid. Water apparently removes inorganic ions from the interior of the modified cells without hemolysis as judged by examining the wash supernatant and the washed cells for their chloride content. Tests with silver nitrate on the final water supernatant have failed to demonstrate the presence of chloride ions. Similar tests done on acid digests of 3 ml of water-washed, packed, formalized red cells also failed to demonstrate the presence of chloride ions. The same quantity of normal red cells contains on the average 0.156 milliequivalents of chloride ions,² an amount readily detected in control tests by the technic used.

Upon standing in contact with water for 12 hours or more the water-washed formalized cells released a yellowish brown pigment to a slight degree into the water. The pigment probably was modified hemin, since it contained iron, reacted negatively for protein with Millon's, Hopkins-Cole, and biuret reagents, the xantho-protein test, and trichloroacetic acid, and in addition, was non-dialyzable. The released pigment became noticeable in the clear supernatant only when the concentration of the cells in the suspension was high.

Washed formalized erythrocytes were found to adsorb and elute influenza A virus hemagglutinating activity in a manner indistinguishable from the action of normal red cells as described by Hirst.³ Suspensions of influenza virus also caused agglutination of the formalized cells just as they agglutinate normal red cells.⁴ Therefore, formalized red cells may replace normal erythrocytes in ti-

trational tests for viral hemagglutinating activity. However, the formalized cells could not be adapted to the Salk pattern technic⁵ since it was observed that these cells agglutinate spontaneously at the bottom of the tube. The turbidometric technic as devised by Hirst⁶ or as modified to use the Klett-Summerson photoelectric colorimeter,⁷ has been employed successfully to titrate viral activity using formalized cells. Due to the greater optical opacity and the slightly greater sedimentation rate of the modified cells as compared with the normal cells, the recorded procedures require slight modification for optimal results.

For the quantitative studies of the adsorption or elution of influenza virus by erythrocytes, formalized cells can be used advantageously in place of normal cells. Formalized cells were found to pack more quickly and firmly than normal cells upon centrifugation so that greater accuracy was obtained in preparing suspensions. Due also to the firm packing, supernatant solutions were more completely removed without loss of the sedimented cells than under comparable conditions with normal erythrocytes. No release of intracellular constituents to any appreciable degree occurs to complicate analyses of the supernatant liquid if the time of reaction is kept moderately brief and if the cells are washed with water previously. As indicated, a stock suspension of the modified cells may be prepared and used over a period of months with assured constancy of concentration and reactivity.

However, there are several disadvantages to the use of formalized red cells. Due to their cohesiveness the packed cells could not be drawn into a pipette without some previous mixing with a liquid diluent. To prepare a suspension of known concentration, a batch of packed cells, entirely free of supernatant,

³ Hirst, G. K., *J. Exp. Med.*, 1942, **76**, 195.

⁴ Hirst, G. K., *Science*, 1941, **94**, 22.

⁵ Salk, J. E., *J. Immunol.*, 1944, **49**, 87.

⁶ Hirst, G. K., and Pickels, E. G., *J. Immunol.*, 1942, **45**, 273.

⁷ Miller, G. L., and Stanley, W. M., *J. Exp. Med.*, 1944, **79**, 185.

¹ Flick, J. A., Sanford, B., and Mudd, S., *J. Immunol.*, in press.

² Peters, John P., *Body Water, the Exchange of Fluids in Man*, Springfield, Ill., Charles C. Thomas, 1935.

was mixed with a known volume of diluent and the total volume of the resulting suspension measured. The concentration of the cells present was computed from the difference in the two volume measurements. Formalized red cells possessed negligible buffering power while normal red cells are known to exert a buffering action in suspension.⁸ Probably due to modification of protein amino groups among others by the formaldehyde, the constituents of the cells could no longer appreciably unite

⁸ Bodansky, M., *Introduction to Physiological Chemistry*, New York, John Wiley & Sons, Inc., 1938, 4th edit.

with hydrogen ions. In the absence of a buffered diluent it was found difficult to maintain the suspension of cells at a constant pH. Finally, while sedimented normal red cells can be resuspended rather easily, suspensions of formalized red cells, upon sedimenting, formed a mass of almost rubbery consistency and required vigorous stirring to resuspend the cells thoroughly. These disadvantages attending the use of formalized red cells are of minor importance and are greatly outweighed by the advantages of red cells stabilized by treatment with 10 to 50% formalin for several days.

16512 P

Practical Method for Determination of Serum Streptomycin Level Using "*Donovania granulomatis*" as Test Organism.*

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(Introduced by G. Lombard Kelly.)

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Following the successful cultivation of "*Donovania granulomatis*" *in vitro* in fresh yolk medium by Dienst, Greenblatt and Chen,¹ the technique for propagating this organism has been made comparable in simplicity to those used for ordinary bacteria. With the demonstration of the high degree of susceptibility of "*Donovania granulomatis*" to streptomycin,^{2,3} it was conceived that this heretofore hard-to-culture micro-organism might be used in streptomycin assay. As a result of a series of experiments the following procedures have been evolved, which we consider as being simple and accurate enough for any clinical or bacteriological laboratory

to use. The principle of this method is based upon the comparison of the least amount of serum to a known quantity of streptomycin that has to be present in the culture medium in order to inhibit the growth of "*Donovania granulomatis*."

The fresh yolk medium is prepared in the following manner: Yolk is removed aseptically from fresh eggs and diluted 1:1 with sterile saline. A base medium containing 1% Bacto peptone, 0.3% Bacto tryptone, 0.3% dextrose, 0.2% sea salt, and 0.12% agar is prepared and adjusted to pH 7.3. The base medium is then sterilized and allowed to cool to 50-60°C before adding an equal portion of the diluted yolk. Four cc of this medium is placed in each of a series of 8 test tubes. The serum to be assayed is diluted with sterile distilled water to 1:1, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, and 1:128. To the first tube of yolk medium is added 1 cc of the 1:1 dilution of serum, to the second tube is added 1 cc of the 1:2 dilution of serum, and so on, so that the final volume of each

* Aided by a research grant from the United States Public Health Service.

¹ Dienst, R. B., Greenblatt, R. B., and Chen, C. H., accepted for publication by the *Am. J. Syph., Gonorr., and Ven. Dis.*

² Greenblatt, R. B., Kupperman, H. S., and Dienst, R. B., *PROC. SOC. EXP. BIOL. AND MED.*, 1947, **64**, 389.

³ Rake, G., and Dunham, V., *Am. J. Syph., Gonorr., and Ven. Dis.*, 1947, **31**, 610.

tube is 5 cc. The serum and the medium are thoroughly mixed by vigorous shaking. Then 0.1 cc of a 4-day-old culture of "*Donovania granulomatis*" is added to each tube and again mixed well by shaking. These tubes are incubated at 37°C and on the fourth day smears are made from each and stained with Wright's stain and examined under oil immersion lens for the presence of the Donovan micro-organism. The tube containing the least amount of serum that inhibited the growth of the organism is regarded as the endpoint.

Calculation. The least amount of streptomycin that will inhibit the growth of the organism has been found by a similar pro-

cedure to be 0.075 γ /cc. Therefore, the serum streptomycin level is equal to $0.075 \times \text{Serum Dilution} \times \text{Total Volume of Medium} \times 2$. For example, if the endpoint of the assay is tube 5, which contains a serum diluted to 1:16, the serum streptomycin level is $0.075 \times .16 \times 5 \times 2 = 12 \gamma/\text{cc}$.

With this simple method, a serum level of 0.75 γ /cc to 48 γ /cc can be determined. This range embraces the serum levels that are commonly encountered in streptomycin therapy. However, with slight modifications, such as using undiluted serum as the first tube and diluting the serum further, one is able to determine a streptomycin level from 0.375 γ /cc to any level higher than 48 γ /cc.

16513

Electron Microscopic Observations on *Pseudomonas aeruginosa* Bacteriophage.

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Isolations of bacteriophages active for *Ps. aeruginosa* (*B. pyocyaneus*) have been reported on a few occasions only. For a time the iridescent erosions which may appear in pyocyaneus colonies were interpreted as manifestations of bacteriophage activity.¹⁻³ It has now been established, however, that these iridescent changes are unrelated to phage activity and that bacteriophagy among pyocyaneus bacilli is not unlike that observed in other species of bacteria.⁴⁻¹¹ Fastier¹²

has recently reported what he erroneously believed to be the first isolation of a pyocyaneus bacteriophage. Between 1930 and 1934 Schultz and Sheets¹³ isolated 4 pyocyaneus bacteriophages which are distinguishable from each other and from the one which is the subject of this report.

The present report is based on a strain isolated by one of us (E.W.S.) in 1943 from the exudate of an extensive third degree burn heavily infected with *Ps. aeruginosa*. It bears our number "Phage 238" and is distinguishable from our earlier strains (Phages 75, 139, 177 and 195)¹³ by its higher lytic titer and

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¹ Hauduroy, P., and Peyre, E., *C. R. Soc. biol.*, 1923, **88**, 688.

² Hadley, P., *J. Infect. Dis.*, 1924, **34**, 260.

³ Rabinowitz, Genia, *J. Bact.*, 1932, **28**, 237.

⁴ Quiroga, R., *C. R. Soc. biol.*, 1923, **88**, 363.

⁵ Combiesco, D., and Magheru, Alice, *C. R. Soc. biol.*, 1923, **88**, 912.

⁶ Pons, R., *C. R. Soc. biol.*, 1923, **89**, 77.

⁷ Okuda, S., *Arch. f. Hyg.*, 1923, **92**, 109.

⁸ Lode, A., *Arch. f. Hyg.*, 1923, **93**, 267.

⁹ Lisch, H., *Centralbl. f. Bakt.*, I Orig., 1924, **93**, 421.

¹⁰ Pesch, K. L., and Sonnenschein, C., *Klin. Wchnschr.*, 1925, **4**, 1585.

¹¹ Asheshob, I., *C. R. Soc. biol.*, 1926, **95**, 1029.

¹² Fastier, L. B., *J. Bact.*, 1945, **49**, 633; **50**, 301.

¹³ Schultz, E. W., and Sheets, Grace, unpublished observations.

wider range of activity for strains of pyocyanus bacilli. It proved highly active for almost all of 75 "colony strains" cultured from the lesions of this patient over a period of 6 months. There was little tendency on the part of the organisms in the lesions to acquire resistance to the phage, even after its application in liberal amounts as a possible therapeutic measure. It induces complete (4+) lysis of young broth cultures and produces typical plaques on solid media. Secondary growths tend to appear slowly. Filtrates usually titer 10^{-8} . The plaques produced on solid media tend to fall into two groups—large and small, the former measuring about 5 mm in diameter, the latter seldom more than 1 mm. The small plaques appear later than the large ones. Attempts to segregate the components responsible for each of these plaques seem to have been wholly successful. Based upon ultra-filtration end-points with Elford's gradacol membranes, the component responsible for the large plaques appears to be definitely smaller (18-27 $m\mu$) than the component responsible for the small plaques (52-78 $m\mu$). The observations here reported seem to apply only to the larger of the two components (to the "small plaque" phage), the smaller of the two not having been identified in electron micrographs.

Electron microscopic observations on bacteriophages (bacterial viruses) active for certain species of bacteria have already been reported.¹⁴⁻²³ Except for a preliminary report of these observations,²⁴ none have been reported on the structure of pyocyanus bacteriophage. A sperm-like form has characterized most of those reported, but differences have also been observed.^{25,26} It seems desirable therefore that observations on various bacteriophages be recorded.

Procedure. The lysate was produced in a synthetic medium consisting of asparagin (1%), NaCl (0.5%), $MgSO_4$ (0.2%) and KH_2PO_4 (0.1%) in distilled water, adjusted to pH. 6 with NaOH. This medium proved more serviceable than broth. Both the organisms and phage were seeded to the medium in the small amounts customarily employed in setting up such tests, along with the usual

controls. The lysed culture was filtered through an L₃ Chamberland candle and 5 cc of the filtrate was then transferred to a chemically clean, sterile centrifuge tube. Enough growth from an 18 to 24 hour agar culture of the homologous organism was then transferred to the filtrate, with a platinum loop, to make it barely cloudy to the naked eye. This mixture was incubated for 5 to 30 minutes at 37°C, then chilled with cracked ice and centrifuged at 2500 r.p.m. for 30 minutes in a refrigerated angle centrifuge at 4°C. The sediment was resuspended in 1 cc of chilled distilled water and again centrifuged. The sediment was now resuspended in 0.5 cc of a chilled 10^{-5} dilution of Zephiran chloride, a surface tension depressant, in distilled water. After slow centrifugation to remove the larger aggregates, the supernatant was transferred to the specimen holder by means of a capillary pipette or platinum loop. The procedure from here on was that ordinarily followed in electron microscopy. A

¹⁴ Ruska, H., *Naturwissenschaft*, 1940, **28**, 45; 1941, **29**, 367.

¹⁵ Pfankuch, E., and Kausche, G. A., *Naturwissenschaft*, 1940, **28**, 46.

¹⁶ Luria, S. E., and Anderson, T. F., *Proc. Nat. Acad. Sci.*, 1942, **28**, 127.

¹⁷ Luria, S. E., Delbrück, M., and Anderson, T. F., *J. Bact.*, 1943, **46**, 57.

¹⁸ Baylor, M. R. B., Severens, J. M., and Clark, G. L., *J. Bact.*, 1944, **47**, 277.

¹⁹ Sharp, D. G., Taylor, A. R., Hook, A. E., and Beard, J. W., *PROC. SOC. EXP. BIOL. AND MED.*, 1946, **61**, 259.

²⁰ Edwards, O. F., and Wyckoff, R. W. G., *PROC. SOC. EXP. BIOL. AND MED.*, 1947, **64**, 16.

²¹ Baylor, Martha B., and Clark, G. L., *J. Bact.*, 1947, **53**, 49.

²² Lépine, P., Giuntini, J., Croissant, O., and Nicolle, P., *Ann. Inst. Pasteur*, 1947, **73**, 582.

²³ Wyckoff, R. W. G., *PROC. SOC. EXP. BIOL. AND MED.*, 1947, **66**, 42.

²⁴ Schultz, E. W., Thomassen, P. R., and Marton, L., *J. App. Physics*, 1944, **15**, 726 (Abstract of paper presented at meeting of the Electron Microscope Society of America, November 16-18, 1944).

²⁵ Ruska, H., *Ergeb. Hyg., Bakt., Immunitätsforsch. u. exp. Therap.*, 1943, **25**, 437.

²⁶ Delbrück, M., *Biol. Rev.*, 1946, **21**, 30.

few specimens were prepared by pervaporation of 2 liters of filtrate to 10 cc, ultracentrifuging the latter, resuspending the sediment in 2 cc of distilled water, and then proceeding as outlined above. This yielded many more phages per unit area on the specimen holder but a less clean specimen.

Observations. A considerable number of electron micrographs were taken by means of the Stanford electron microscope, described elsewhere.²⁷ Observations were carried out at 70 to 80 ekv. Analysis of a number of electron micrographs of Phage 238 show that at least one of the components, probably the larger of the two referred to, is composed of a head and tail portion, these parts being in much the same drum-stick type of relationship as the spore is to the rod in sporulated tetanus bacilli (Fig. 1). The head of this combination appears to be pentagonal in shape and to contain a dense filamentous structure. The average diameter of the head is 76 to 85 $m\mu$, while the dimensions of the tail are about 150 $m\mu$ by 15 $m\mu$. The form of the filamentous inner structure in the head portion frequently suggests the form of the letter "W" (Fig. 2 and 3). There are, however, some indications that its true projected shape approaches a distorted figure 8; in other words, that it consists of relatively straight longer portions linked together by sharply curved shorter portions. It appears that it is this relatively thick, dense and unyielding filamentous structure within the substance of the head which gives to it its irregular pentagonal shape. This suggests that the different portions of the filament do not lie wholly within one plane. What appears to be a confirmation of this view was obtained when suitably taken electron micrographs were examined stereoscopically. Several stereomicrographs were taken at $\pm 13^\circ 53$ min. tilting angle by means of a special stage employed in the Stanford electron microscope.²⁸ From these observations it appears that the dense filament does not lie in a plane, but forms an apparently continuous loop. This loop appears to be bent in a mid plane, forming an

obtuse angle with the long axis of the loop. Surrounding and stretched between the segments of this filament there is a less dense material which is apparently continuous with the tail portion. The latter possesses about the same density as the former, and may therefore consist of the same material. The density of the tail portion seems to be approximately that of some bacterial flagella. The tail is almost always seen as a straight or uncurved process and seems always to come off the head portion opposite the middle of one of the longer straight sections of the filament, from where, however, it may come off at different angles.

A few apparently intact bacterial cells were observed which showed rounded internal structures about the size of the heads of the phages here described (Fig. 4). Since some of these appear pentagonally shaped, these may be phages located within the cells. Stereomicrographs served to confirm that these bodies are lying inside of the cell boundary or wall. One of the objectives of these studies was to determine, if possible, the host relationships of the phage and its mode of reproduction. These questions were not answered, however. While many apparently intact bacterial cells were observed with phages on or near their surfaces, also many cells which had apparently undergone phage lysis, little was revealed in the micrographs as to where and how the phage is reproduced.

A considerable number of micrographs of specimens prepared from phage untreated cultures of the organisms employed in these studies failed to show the structures here described as pyocyanous phage. A highly resistant lysogenic culture, found among the many "colony strains" originally isolated from the patient, was lost before these studies were undertaken and several attempts to create and isolate a similarly resistant strain by artificial exposures to phage proved unsuccessful. It would be of considerable interest to observe the relationships of the phages produced by highly resistant lysogenic strains.

Three other strains of pyocyanous phages were examined less extensively. A few definitely sperm-shaped structures were iden-

²⁷ Marton, L., *J. App. Physics*, 1945, **16**, 131.

²⁸ Marton, L., *J. App. Physics*, 1944, **15**, 726.

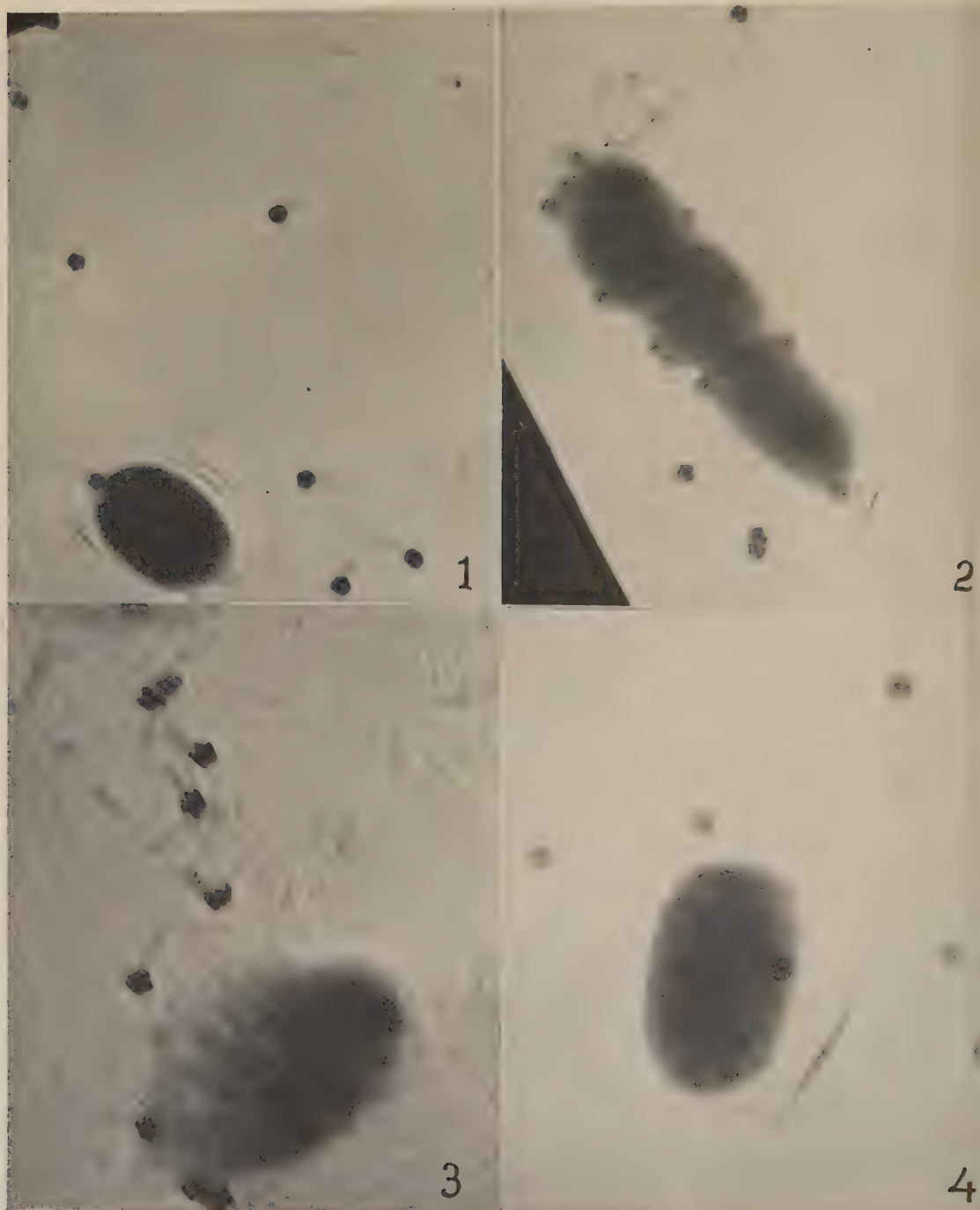


FIG. 1. *Ps. aeruginosa* bacteriophage particles, showing both a head and tail portion. The former is of irregular form due to a rigid internal structure. One of the phage particles is adsorbed to a bacterial cell. (30,000 \times .)

FIG. 2. An underexposed print of a micrograph showing the characteristic W-shaped internal structure in the head portions of the phage particles. Several of the particles are adsorbed to a dividing bacterial cell. (30,000 \times .) The underexposure in making the print was

employed as a photographic artifice for bringing out details of internal structure, but by doing so the tails of the phage particles were lost.

FIG. 3. Enlarged and slightly underexposed print of a micrograph showing the W-shaped dense structure in the head portion of individual particles. (50,000 \times .)

FIG. 4. An underexposed print of a micrograph showing a bacterial cell containing rounded but somewhat irregularly shaped bodies suggesting the pentagonal form of the heads of phage particles. Underexposed phage particles of approximately the same size lie in the vicinity of the bacterial cell. (50,000 \times .)

tified in micrographs of one of these (Phage 139). These appeared to be somewhat smaller than those of the phage here described. No distinctive bodies were identified in micrographs of the two other strains (Phages 75 and 177). A remaining strain (Phage 195)

was not examined, because of an interruption of these studies.

Summary. A sperm-shaped structure was identified with two bacteriophages active for *Ps. aeruginosa*.

16514

Thermostability and Alcohol Solubility of Vi Antigen.

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The present investigation was suggested by an unpublished observation made by one of us several years ago while working on Vi antigens and antibodies. An antiserum prepared against a motile Vi positive typhoid strain, H-484, was adsorbed 3 times with *Salmonella ballerup*. No drop in titer occurred for either O-901, H-484 or its non-motile Vi positive variant, O-484. However, a marked prozone, increasing with the number of adsorptions, developed against O-484 (Table I). When *S. ballerup* was washed in several changes of saline before being used for adsorption, the prozone against O-484 failed to appear. It would seem that the Vi antigen was related to this prozone since it was the only component common to O-484 and *S. ballerup*. The present work is an attempt to explain this reaction.

The growth of *S. ballerup* was removed from plates using 5 ml of saline for every 2 plates. The mixture was shaken several times, centrifuged after 30 minutes and the supernatant fluid used to harvest more plates. The procedure was repeated a third time. To 5.85 ml of the final supernatant fluid was added .15 ml of H-484 antiserum. From this mixture 3 sets of serial dilutions were made

in saline so that the antiserum dilutions ranged from 40 to 40,960 and the supernatant fluid dilutions from 1 to 1,024. To one set was added .1 ml of a saline suspension of antigen O-901, to another H-484 and to the third O-484. For controls the same antigens were added to saline dilutions of H-484 antiserum. In this experiment the controls are comparable to the tests in Table I labeled "before adsorption" and the mixture of supernatant fluid, antiserum and antigen comparable to those labeled "after third adsorption." Tests were set up in duplicate and after 4 hours at 37°C one test was placed at 2°C for about 18 hours and the other at 55°C for the same time. The results of this experiment, except for small differences in titer and degree of inhibition at the different temperatures, were identical with those shown in Table I.

To test further its ability to inhibit agglutination the supernatant was diluted serially from 1 to 1,024 in saline. To a .5 ml portion of each dilution was added .1 ml of that dilution of pure Vi antiserum just sufficient to give complete agglutination of the test strain in a total volume of .7 ml. The tubes were shaken, allowed to stand about 15 minutes and .1 ml of a saline suspension of O-484

TABLE I.
Adsorption of Typhoid H-484 Vi Antiserum with *S. ballerup*.

Test antigen Before adsorption	Serum dilutions										
	40	80	160	320	640	1280	2560	5120	10240	20480	40960
O-901	4	4	4	4	4	4	4	4	3	1	—
H-484	4	4	4	4	4	4	4	4	4	3	1
O-484	4	4	4	4	4	4	4	4	3	1	—
After first ads.											
O-901	4	4	4	4	4	4	4	4	2	±	—
H-484	4	4	4	4	4	4	4	4	3	2	1
O-484	1	2	4	4	4	4	4	4	3	1	—
After third ads.											
O-901	4	4	4	4	4	4	4	3	1	—	—
H-484	4	4	4	4	4	4	4	3	2	1	—
O-484	—	—	—	1	3	4	4	3	1	—	—

4, 3, 2, and 1—degrees of agglutination from complete to weak.

added. As a control antiserum and antigen were added to .5 ml of saline. Tests were incubated at 37°C for 4 hours followed by 18 hours at 2°C. Complete agglutination of the test organism in the control was accompanied by complete inhibition in the lower dilutions of the supernatant. This indicated that free or soluble Vi antigen in the supernatant had adsorbed the Vi antibodies before an effective union could be made with the fixed or cellular Vi antigen. Similar results were obtained with supernatants from the Bhatnagar, Watson Ty 2 and other Vi typhoid cultures using pure Vi antiserum prepared from the first 3 strains.

It was surprising to find almost as good inhibition of agglutination after 18 hours at 55°C as at 37°C in the first experiments since Vi antigen is supposed to be destroyed at 60°C for one hour. To determine the effect of heat on inhibition, saline suspensions of Vi positive organisms were heated to 60°C and in a boiling water bath for from 1 to 3 hours. After centrifugation a clear, fluorescent greenish-yellow "extract" was obtained. These extracts strongly inhibited Vi agglutination, were efficient precipitinogens with pure Vi antisera and produced strong Vi antibodies upon injection into rabbits. Saline suspensions of O-inagglutinable Vi typhoid strains were heated to 100°C for 1 hour and centrifuged. The extract was positive in tests for the presence of Vi antigen while the washed bacterial bodies were O-agglutinable and failed to agglutinate in pure Vi antiserum. Saline extracts from

heavy suspensions of Vi strains heated to 121°C for 3 hours were strongly positive in inhibition tests. These extracts, however, produced only low titering (40 to 80) Vi antisera after prolonged injection into rabbits which confirms the work of Smith.¹

All saline extracts prepared from unheated and particularly heated suspensions of Vi positive strains contained high concentrations of O as well as Vi antigen as shown by precipitin tests with O-901 antisera.

Alcoholic extracts were prepared from *S. ballerup* and Vi typhoid strains by harvesting the growth from plates with absolute ethyl alcohol. The suspensions were placed at 37°C for one or 2 days and shaken frequently. After centrifugation the supernatant was evaporated to dryness at 37°C. The residue was taken up in saline and the insoluble portion removed by centrifugation. To insure the removal of all water soluble material, occasionally the residue was redissolved in absolute ethyl alcohol, centrifuged and the clear supernatant evaporated *in vacuo* over sulphuric acid. These alcohol soluble-saline soluble extracts were strongly positive in all the tests for the presence of Vi antigen, including the production of Vi antibodies.

Saline supernatants, saline heated extracts and alcoholic extracts of a single Vi positive strain varied greatly in their properties, particularly in the concentration of Vi antigen. Saline supernatants showed complete inhibition of Vi agglutination in dilutions of

¹ Smith, E. V. D., *J. Infect. Dis.*, 1938, **63**, 21.

4 to 8, heating from 60°C to 100°C markedly reduced the Vi content and filtering through a Seitz or Berkefeld completely removed it. Saline heated extracts gave complete inhibition in dilutions of 32 to 64, resisted 100°C for 3 hours and produced Vi antisera with titers as high as 2,560. They could be filtered though some loss in Vi content usually occurred. Alcoholic extracts showed complete inhibition in dilutions as high as 512, were thermostable, have been filtered without loss and have produced pure Vi antisera with titers as high as 20,480.

Saline supernatants, saline and alcoholic extracts of O-901 and other cultures lacking Vi were uniformly negative in the tests described to detect Vi antigen.

Discussion. The extreme thermostability (destruction or inactivation in 1 hour at 60°C) commonly assigned to the Vi antigen of *S. typhosa* has been questioned by many investigators. Horgan,² Robertson and Yu,³ Smith,¹ Peluffo,⁴ Luippold,⁵ and others found that the Vi antigen of some strains of typhoid resisted 100°C for varying periods of time up to 1 hour. It is recognized that upon heating the ability of the Vi antigen to (1) cause O-inagglutinability, (2) produce antibodies, (3) agglutinate in antiserum and (4) adsorb antibodies, tends to disappear in the order

listed. This progressive loss in characteristics may indicate that the Vi antigen contains more than one component. Obviously the method used to detect the presence of Vi antigen after heating is important and the present report shows that the material used to ascertain the presence of Vi may be more important than the method.

Heating suspensions of Vi strains seems to release all or a major portion of this antigen from the cell. Free in the menstrem the Vi component is relatively thermostable but its disappearance from the cell could easily be interpreted as destruction. The Vi antigen of Felix and Pitt,⁶ the alpha antigen of Stamp and Stone,⁷ the L antigen of Kauffman⁸ and an antigen of *Paracolonobacterium intermedium*⁹ to be reported later have much in common. They inhibit or interfere with O-agglutination and are more or less widespread in the *Enterobacteriaceae*. Heating releases these antigens from the cell and/or restores O-agglutinability; at least 2 are alcohol soluble.

The relative thermostability and alcohol solubility of the Vi antigen would indicate a possible close relationship to the alcohol soluble heterophile antigens of tissues such as the Forssman, but unlike the latter the former will produce antibodies without the mediation of a conveyor.

² Horgan, E. S., *J. Hyg.*, 1936, **36**, 368.

³ Robertson, R. C., and Yu, H., *J. Path. and Bact.*, 1936, **42**, 53.

⁴ Peluffo, C. A., *Proc. Soc. Exp. Biol. and Med.*, 1941, **48**, 340.

⁵ Luippold, G. F., *Am. J. Pub. Health*, 1946, **36**, 15.

⁶ Felix, A., and Pitt, R. M., *J. Path. and Bact.*, 1934, **38**, 409.

⁷ Stamp, L., and Stone, D. M., *J. Hyg.*, 1944, **43**, 266.

⁸ Kauffmann, F., *J. Immunol.*, 1947, **57**, 71.

⁹ *Bergey's Manual*, Sixth Edition, 1948.

Streptomycin Resistant Variants Obtained from Recently Isolated Cultures of Tubercle Bacilli.*

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One of the most important limitations upon the use of streptomycin in the treatment of tuberculosis is the rapidity with which tubercle bacilli which have become resistant to bacteriostatic action of streptomycin develop in many patients undergoing treatment with the drug. The development of such drug-resistant forms of bacteria is generally believed to be brought about by the selection of resistant mutants present in the original bacterial population.

Pyle¹ has shown that the tubercle bacilli isolated directly from patients prior to the institution of streptomycin therapy are composed of bacterial cells which do not have a uniform susceptibility to the bacteriostatic action of streptomycin. According to Pyle, the streptomycin resistant population of tubercle bacilli in patients undergoing treatment with streptomycin gradually increases as administration with streptomycin continues. Apparently, the more susceptible tubercle bacilli are retarded in their growth, whereas the more resistant ones are not. Once a predominantly resistant population develops, the tuberculous process is refractory to streptomycin therapy.

Young, Williston, Feldman and Hinshaw,² Middlebrook and Yegian³ and Williston and Young⁴ have shown that cultures of virulent tubercle bacilli, including the H37Rv strain, will become resistant to the bacteriostatic action of streptomycin following serial transfer in media containing small amounts of streptomycin.

Vennesland, Ebert, and Bloch⁵ later reported the isolation of streptomycin resistant variants of the virulent laboratory strain of *M. tuberculosis* H37Rv after a single exposure of large numbers of tubercle bacilli to high concentrations of streptomycin. These variants were resistant to more than 10,000 times the concentration of streptomycin necessary to inhibit the growth of the original culture. There is a possibility that these streptomycin resistant variants may play a large role in the development of the high degree of resistance frequently observed in cultures obtained from patients under treatment with streptomycin. The demonstration that recently isolated strains of tubercle bacilli would produce these variants, would tend to strengthen this possibility.

Methods. Streptomycin in the following concentrations, 1000.0, 500.0, 100.0, 50.0, 25.0, 12.5, 6.25, 3.12, 1.56, 0.78, 0.39, 0.19, and 0.095 μ g per ml, was incorporated in Herrold's glycerin egg agar medium⁶ cooled to 45°C. The medium was then tubed in 20 by 125 mm screw top test tubes, and was allowed to harden in the form of slants. Fine suspensions of the recently isolated cultures of tubercle bacilli were prepared by grinding, as previously described,⁷ and a slant of medium of each streptomycin concentration was inoculated by pipet with 0.1 to 0.25 mg wet weight of tubercle bacilli. In some cases inoculation was made directly from cultures

* This investigation was supported (in part) by a research grant from the Division of Research Grants and Fellowships of the National Institute of Health, U. S. Public Health Service.

¹ Pyle, M. M., *Proc. Staff Meetings, Mayo Clinic*, 1947, **22**, 465.

² Young, G. P., Williston, E. H., Feldman, W. H., and Hinshaw, H. C., *Proc. Staff Meetings, Mayo Clinic*, 1946, **21**, 126.

³ Middlebrook, G., and Yegian, D., *Am. Rev. Tuberc.*, 1946, **53**, 553.

⁴ Williston, E. H., and Young, G. P., *Am. Rev. Tuberc.*, 1947, **55**, 536.

⁵ Vennesland, K., Ebert, R. H., and Bloch, R. G., *Science*, 1947, **106**, 476.

grown in Dubos medium.⁷ Following inoculation, the cultures were incubated at 37°C for a period of 6 weeks. At the end of 2 weeks, a reading was made of the least amount of streptomycin which completely inhibited the growth of the culture. This value was recorded as the sensitivity of the culture as a whole to streptomycin. At weekly intervals thereafter, the tubes were examined for the presence of colonies of tubercle bacilli that may have grown out on the higher concentrations of streptomycin. When found, these were transferred to fresh medium and, in most cases, retested for their sensitivity to streptomycin both on solid and liquid medium. Again the least amount of streptomycin which completely inhibited growth was recorded as the sensitivity of these cultures to streptomycin.

The 57 recently isolated cultures used in this study were obtained from various hospitals and sanatoria in the Middle West, and came from patients either under streptomycin treatment or from patients for whom streptomycin therapy was contemplated.

Results. All the cultures of tubercle bacilli showed profuse growth within two weeks on the Herrold's control medium which did not contain streptomycin. At this time, depending upon the sensitivity of the culture to streptomycin, there was also growth on some of the tubes of medium containing the lower concentrations of streptomycin. As the time of incubation lengthened, growth would usually appear on tubes containing the next one or two higher concentrations of streptomycin. This later growth usually consisted of only a few scattered colonies. These, by test, were found to be organisms of slightly greater resistance to the bacteriostatic action of streptomycin than the majority of the cells in the original culture. These findings confirmed the report of Pyle¹ that cultures of tubercle bacilli are not composed of cells having a uniform sensitivity to streptomycin.

TABLE I.
Streptomycin Resistant Variants Obtained from
Cultures Isolated from Patients Who Had Not
Received Streptomycin Therapy.

Strain No.	Streptomycin sensitivity of culture in Herrold's Medium	Conc. of streptomycin on which streptomycin resistant variant was found
H37Rv	1.56	12.5
H37Rv	1.56	6.25
H37Rv	1.56	50.0
1	1.56	6.25
2	1.56	6.25
3	6.25	50.0
4	1.56	25.0
4	1.56	12.5
5	1.56	1000.0
5	1.56	500.0
5	1.56	100.0
5	1.56	50.0
6	1.56	12.5
7	3.12	12.5
8	3.12	100.0
9	3.12	25.0

The sensitivity of these organisms, however, was no more than 2 to 4 times less than that of the majority of the cells in the culture. Patients being treated with streptomycin, however, are known to discharge tubercle bacilli that are several thousand fold more resistant to the bacteriostatic action of streptomycin.

Examination of the tubes after 4 weeks of incubation frequently revealed single isolated colonies of tubercle bacilli growing on much greater concentrations of streptomycin than the rest of the culture. These colonies seldom appeared within less than 4 weeks of incubation and were usually quite small; the colonial morphology, however, was similar to that of the rest of the culture.

Table I shows the number of these streptomycin resistant variants isolated from 9 out of 14 (64.3%) cultures of tubercle bacilli obtained from patients who had never received streptomycin. Also included is the virulent laboratory strain, H37Rv. The majority of these variants, after subculture, were retested for their streptomycin sensitivity; in all cases they were resistant to a concentration of streptomycin which was equal to or greater than the concentration in the agar medium upon which they were originally isolated.

⁶ Herrold, R. D., *J. Infect. Dis.*, 1931, **48**, 236.

⁷ Youmans, G. P., and Karlson, A. G., *Am. Rev. Tuberc.*, 1947, **55**, 529.

⁸ Dubos, R. J., and Davis, B. D., *J. Exp. Med.*, 1936, **83**, 409.

TABLE II.
Streptomycin Resistant Variants Obtained from
Cultures Isolated from Patients Who Had Re-
ceived Streptomycin Therapy.

Strain No.	Streptomycin sensitivity of culture in Herrold's Medium	Conc. of streptomycin on which streptomycin resistant variant was found
10	1.56	50.0
10	1.56	12.5
11	1.56	12.5
12	1.56	12.5
13	3.12	50.0
13	3.12	25.0
14	1.56	500.0
14	1.56	100.0
15	6.25	50.0
15	6.25	25.0
16	6.25	100.0
17	6.25	100.0
17	6.25	50.0
18	3.12	50.0
18	3.12	12.5
19	3.12	500.0
19	3.12	100.0
19	3.12	50.0
19	3.12	25.0
19	3.12	12.5
20	1.56	50.0
20	1.56	12.5
21	1.56	50.0
22	12.50	100.0
23	1.56	25.0
24	1.56	25.0
24	1.56	12.5
25	3.12	1000.0

Of the 43 cultures obtained from patients following the institution of streptomycin therapy, streptomycin resistant variants were isolated from 16 (37.1%). These are shown in Table II. All of these cultures, with the exception of number 22 would be considered relatively sensitive strains on the basis of conventional streptomycin sensitivity tests. All of the streptomycin resistant variants which were retested for sensitivity to streptomycin

were again found to be resistant to a concentration of streptomycin which was equal to or greater than the concentration contained in the medium upon which they were originally isolated. Frequently they were considerably more resistant. For example, 5 streptomycin resistant variants of strain No. 19 were found upon the following concentrations of streptomycin: 500.0, 100.0, 50.0, 25.0, and 12.5 μ g per ml. When retested, these variants were found to be sensitive to the following concentrations of streptomycin, respectively; greater than 1000.0, greater than 1000.0, 100.0, 500.0, and 12.5 μ g per ml.

Discussion. The presence of streptomycin resistant variants of tubercle bacilli in such a high proportion of recently isolated strains of tubercle bacilli, suggests strongly that these variants may play a role in the development of the streptomycin resistance which occurs in cultures of tubercle bacilli isolated from patients having had prolonged treatment with streptomycin. As yet, sufficient data are not available to determine whether those patients, whose cultures which were isolated prior to the administration of streptomycin and which subsequently showed the presence of streptomycin-resistant variants, will be more likely to develop resistant cultures upon streptomycin therapy. This may be of importance because if such a correlation does exist, it might provide an indication, before the beginning of therapy or early during therapy, as to which patients are likely to develop resistant strains.

Conclusions. Streptomycin resistant variants were obtained from 25 out of 57 (43.5%) recently isolated strains of tubercle bacilli.

Giant-Cell Inclusions in Cicatrizing Enteritis.

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The cause of cicatrizing enteritis (regional ileitis) remains unknown. Similar lesions have been produced experimentally by injections of sclerosing solutions into the mesenteric lymphatics of dogs.¹ Various lipids introduced parenterally provoke granuloma formation such as characterizes cicatrizing enteritis.^{2,3} In this disease giant cells in the granulomas stain for fat,⁴ and in addition occasionally contain crystalloid inclusions.^{5,6} As part of a study of etiologic factors in cicatrizing enteritis, investigation of the origin and significance of these inclusions appeared worthwhile.

Two specimens among a group of 138 cases of cicatrizing enteritis contained numerous enough inclusions to make detailed study possible. The patients were women aged 19 and 26 years, who had portions of the small intestine removed surgically. The gross appearance of the resected specimens was characteristic of cicatrizing enteritis, with pronounced edematous and inflammatory thickening of the intestinal wall and mesentery. A fine stippled gray line near the serosal surface of each sectioned bowel marked the location of most of the inclusions. Histologically, there were many of these colorless rounded objects with smooth surfaces within the giant cells, measuring up to about 45 μ in

diameter, and varying two-fold or more in size (Fig. 1). Some showed concentric laminations. They did not stain with any of the usual reagents, were strongly doubly refractile in polarized light, and remained unaffected by heat sufficient to char the tissue. Regional lymph nodes contained similar inclusions. They were not found extracellularly, and did not resemble any living organism.

Physical and chemical properties investigated were identical for the inclusions of both cases. The objects were slowly soluble in distilled water over a period of approximately 48 hours. They were easily soluble in dilute hydrochloric acid and ammonium chloride, insoluble in sodium hydroxide, acetic acid, glycerine, alcohol and dioxane. The refractive index by the immersion method was 1.65 or higher.

By means of a micropipette many of these objects were freed from paraffin tissue sections under polarized light and dissolved in small amounts of water. Qualitative tests made separately on the two cases by the micromethods of Chamot and Mason⁷ gave strong reactions for calcium. Attempts to identify the anions by similar methods were unsuccessful. Presence of bismuth and silicates appeared to be excluded by these experiments.

The origin of the inclusions has been frequently discussed.⁴ Their localization in the subserosa of the intestine and its regional lymph nodes argues against a dietary source. In morphology and chemical properties they resemble the psammoma bodies of meningiomas. Bailey's theory that certain types of cell necrosis attract calcium salts⁸ may serve to explain both psammoma formation

¹ Reichert, F. L., and Mathes, M. E., *Ann. Surg.*, 1936, **104**, 601.

² Pinkerton, H., quoted in Homans, J., and Hass, G. M., *New England J. Med.*, 1933, **209**, 1315.

³ Geyer, R. P., Mann, G. V., Young, J., Kinney, T. D., and Stare, F. J., *J. Lab. and Clin. Med.*, 1948, **33**, 163.

⁴ Warren, S., and Sommers, S. C., *Am. J. Path.*, 1948, **24**, 475.

⁵ Moscheowitz, E., and Wilensky, A. O., *Am. J. M. Sci.*, 1923, **166**, 48.

⁶ Manson-Bahr, P., *The Dysenteric Disorders*, Williams & Wilkins Co., Baltimore, 1943, 629 pp., p. 517.

⁷ Chamot, E. M., and Mason, C. W., *Handbook of Chemical Microscopy*, J. Wiley & Sons, London, 1940, Vol. II.

⁸ Bailey, O. T., *Arch. Path.*, 1940, **30**, 42.

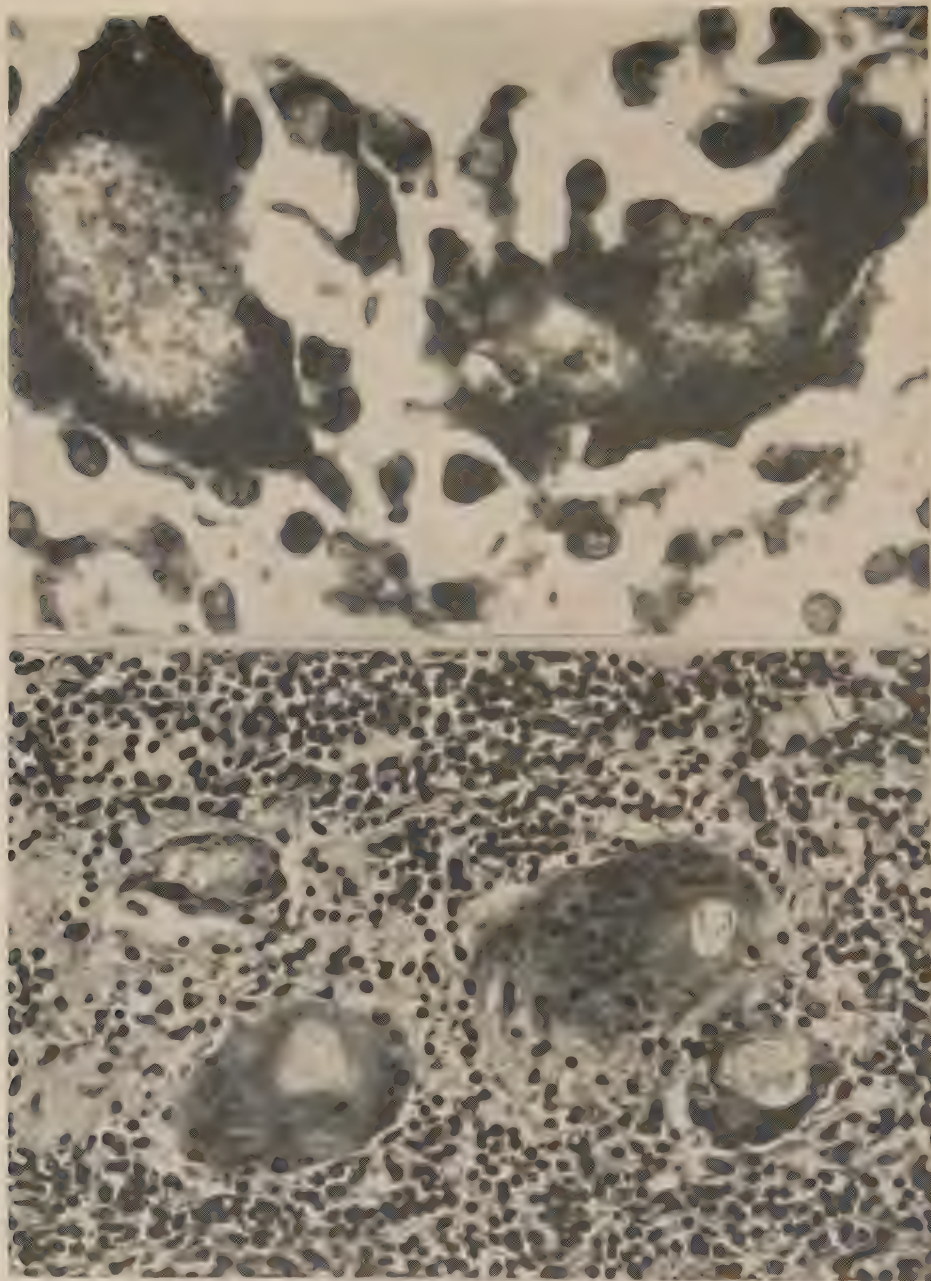


Fig. 1.
Several giant cells in cicatrizing enteritis are shown with laminated refractile inclusions. Eosin methylene blue. $\times 375$.

Fig. 2.

Two giant cells, one containing a central area of cytoplasmic concentration suggesting an early stage of inclusion formation. Iron hematoxylin. $\times 1500$.

and the development of these inclusions. An early stage of an inclusion is suggested by a focal abnormal concentration of cytoplasm of

a giant cell observed in cicatrizing enteritis (Fig. 2). Absence of organic material from the fully developed inclusions is responsible

for their failure to take the usual stains.

These giant-cell inclusions are therefore considered to be a byproduct of granulomatous inflammation in cicatrizing enteritis.

Summary. Inclusion bodies in the giant cells of 2 cases of cicatrizing enteritis (regional ileitis) were investigated. By physical and chemical methods they were found to be in-

organic calcium salts. The anions were not identified. Their origin is attributed to focal abnormalities of the cytoplasm of giant cells found in this type of granulomatous inflammation, which attract calcium salts. In their development they somewhat resemble psaminoma bodies.

16517

Effect of Strophanthin and Quinidine upon Conduction and Electrical Systole (Q-T Interval) of the Rabbit Heart.*

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Previous studies from this laboratory^{1,2} noted the effects on conduction of varying rates of stimulation, and of the use of strophanthin and quinidine, upon the isolated rabbit ventricles. Intraventricular conduction times (QRS widths) increased markedly at extremely high (300 and over) rates of stimulation. Strophanthin, and particularly quinidine, prolonged the QRS intervals at all rates of ventricular contraction.¹ The electrical systole or Q-T interval showed a more gradual decrease with increasing ventricular contraction rates. Small doses of strophanthin, of the order of 0.006 mg/kg, in general prolonged the Q-T interval; larger doses, *e.g.* 0.018 mg/kg, uniformly shortened it,² in line with its known clinical effect. Quinidine consistently prolonged the ventricular electrical systole or the Q-T interval, as it delayed intraventricular conduction.

The isolated rabbit ventricles were a useful instrument of study for two reasons. First, they could be driven at much faster rates than the whole heart. Secondly, we

were able to elicit the effects of various drugs upon the ventricular musculature without the complicating interconnected influences upon the atria and the A-V junctional tissues. The following presentation is of similar studies made with the isolated whole rabbit heart.

Method. The procedure was essentially the same as in the previous experiments. The perfusion fluid best adapted for the whole heart we found to be the one used by Calder³ under similar conditions. The atria were left on and the electrodes of the thyrotron stimulator applied usually to the right atrium. The electrocardiographic records were made as before, approximately at 10 minute intervals after the introduction of the drugs into the rubber tube leading to the aortic cannula. Control measurements similarly spaced and rest periods between salvos of rapid stimulation excluded the effects of fatigue. Oxygenation and a constant 37°C temperature of the perfusing fluid were provided as before by means of continuous bubbling of 5% CO₂ in oxygen and a heated water jacket respectively.

Results. Effect of varying rates. Lengthening of the P-R interval occurred, gradually as a rule, at increasingly fast rates of stim-

* Supported, in part, by a grant from the Sandoz Chemical Works, Inc.

¹ Decherd, G., and Ruskin, A., *Proc. Soc. Exp. Biol. and Med.*, 1946, **63**, 114.

² Ruskin, A., and Decherd, G., *Proc. Soc. Exp. Biol. and Med.*, 1946, **63**, 117.

³ Calder, R. M., *Proc. Soc. Exp. Biol. and Med.*, 1947, **65**, 76.

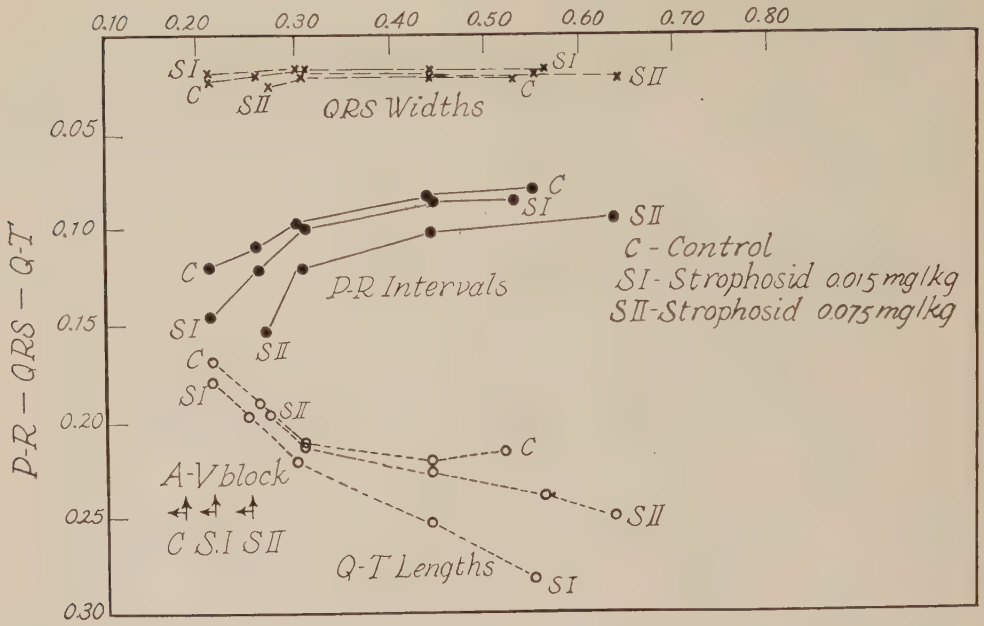


FIG. 1.

Effects of a small, and later a larger, dose of strophanthin upon the P-R, QRS, and Q-T intervals of the electrocardiogram of the isolated rabbit heart at various cycle lengths (R-R intervals (abscissae in seconds)).

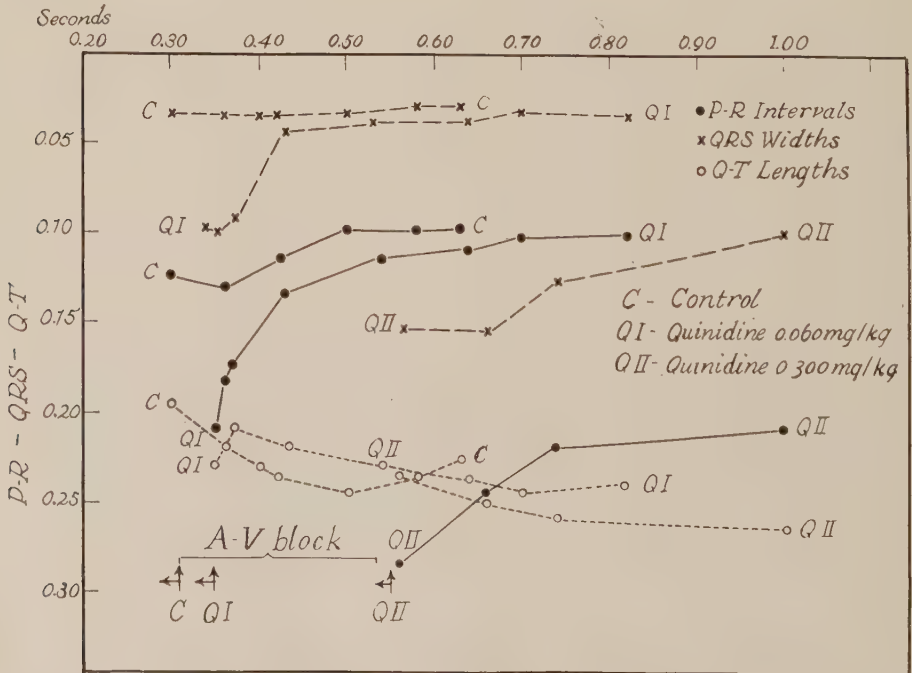


FIG. 2.

Effects of a small, and later a larger, dose of quinidine upon the P-R, QRS, and Q-T intervals of the electrocardiogram of the isolated rabbit heart at various cycle lengths (R-R intervals (abscissae)).

ulation. Great prolongation of the P-R interval at rapid rates of stimulation before second degree A-V block intervened was noted infrequently. The QRS width decreased very slightly before dropped beats occurred under similar circumstances of increasingly rapid stimulation (Fig. 1 and 2). These results contrast with the much steeper recovery curves of intraventricular conduction when ventricles are directly stimulated at much faster rates.¹ Nor did we usually observe any transient improvement in A-V conduction over a short range of rapid rates, causing a notch in the recovery curves, such as we observed in our previous studies of intraventricular conduction.¹ That the QRS widths likewise failed to shorten and then increase markedly at rapid rates of stimulation may be explained on the basis of the strongly limiting factor of A-V block upon these rates. QRS widening and aberration was again noted in the second, third and sometimes fourth complexes of groups produced by sudden rapid rates of stimulation, provided these rates approached those possible by direct ventricular stimulation.¹ Corresponding widening of P-R intervals, even to dropped beats, occurred at the beginning of groups of complexes produced by a series of rapid stimuli with relatively short rest periods in between. This phenomenon, presumably dependent on fatigue and increased relative refractoriness, has been noted in experimental A-V Wenckebach block by Scherf⁴ and in patients with paroxysmal tachycardia by Decherd *et al.*⁵

The generally inverse relations of the Q-T interval to cycle length were seen in this (Fig. 1 and 2) as in our previous study.¹ That they deviate from the straight line relations predicated by Robb,⁶ and by Schlamowitz,⁷ may be explained on the bases of the special conditions of our experiments, involving very

slow as well as very fast cardiac rates, at times beyond physiological limits, and elimination of extracardiac reflex nervous factors.

Effects of Strophanthin (Strophosid, Sandoz). The well-known slowing of A-V conduction by digitalis and strophanthin glucosides was substantiated by our study (Fig. 1). P-R intervals were prolonged at all rates of stimulation in proportion to the dose employed. QRS prolongation was noted especially at rapid rates of stimulation and with the larger doses of the drug (Fig. 1). These effects were more marked in our previous study, employing the ventricles only and at faster rates. Small doses of strophanthin caused prolongation of the Q-T interval and this was usually reversed by larger doses (Fig. 1); this latter shortening is comparable to its characteristic clinical effect⁹; prolongation with small doses has been noted in the isolated frog heart.⁸ We have been able to demonstrate similar effects employing other glucosides (Cedilanid (Sandoz), Scillaren-B (Sandoz), and digitoxin). The usual effects upon the S-T segments were also seen.

Effects of quinidine. The effects on conduction upon addition of quinidine to the perfusate were classical, *i.e.* A-V and I-V conduction were both slowed at all rates of stimulation in proportion to the dose administered (Fig. 2). The effects upon the Q-T interval were not clear-cut, however. Slight prolongation, at most, was noted with larger doses (Fig. 2). At times paradoxical shortening was obtained despite definite delays in conduction as well as in the absolute refractory period. This contrasts sharply with the prolongation noted in the ventricular preparations¹ and in clinical usage of the drug.

Comment. The recovery curves of A-V and intraventricular conduction parallel closely those previously published for the two loci of conductivity by Lewis and Master¹⁰ and by us¹ respectively. Beyond a certain rate

⁴ Scherf, D., *Wien. Arch. inn. Med.*, 1939, **18**, 403.

⁵ Decherd, G. M., Herrmann, G. R., and Schwab, E. H., *Am. Heart J.*, 1943, **26**, 446.

⁶ Robb, J. S., and Turman, W. G., *Am. J. Med. Sc.*, 1947, **214**, 180.

⁷ Schlamowitz, I., *Am. Heart J.*, 1946, **31**, 329.

⁸ Clark, A. J., and Mines, G. R., *J. Physiol.*, 1913, **47**, VII.

⁹ Kisch, B., *Strophanthin*, Brooklyn Medical Press, 1944.

¹⁰ Lewis, T., and Master, A. M., *Heart*, 1925, **12**, 209.

of cardiac beating the conduction time is more and more prolonged as the recovery period shortens.

The results are in general confirmatory of what is known about the cardiac effects of strophanthin and quinidine, with the possible exception of the Q-T interval modifications by quinidine. Both drugs, especially quinidine, prolong atrio-ventricular, as well as intraventricular conduction, more so at rapid rates of cardiac beating. These are presumably direct muscular effects in our experiments, unless the amputated nerve endings are also affected (?). The recovery curves are markedly depressed downward and to the right, indicating prolongation of the relative and absolute refractoriness of the atrio-ventricular as well as the ventricular musculature. The slower the recovery of conductivity, the longer the period of relative refractoriness, the greater the incidence of partial A-V and Wenckebach types of block. This is our theoretical prediction¹¹ as well as the actual occurrence in our data.¹² Both strophanthin and quinidine, thus, predispose to Wenckebach

(and Mobitz) types of A-V block, though they are found also frequently enough in our control curves at rapid cardiac rates. These findings substantiate the known clinical effects of the two drugs.

Rapid rates of stimulation of the auricles, up to 300 per minute, usually failed to elicit paroxysmal ventricular tachycardia or fibrillation, seen in the faster stimulated ventricular preparations,¹ typically after marked QRS aberration. Ventricular ectopic beats, with bigeminy, occurred not infrequently, but runs of them were rare, nor were they apparently influenced by strophanthin or quinidine in these, as in the ventricular,¹ preparations of rabbit hearts.

Summary. Recovery curves of conductivity of the A-V node and ventricles for the isolated rabbit heart driven at various rates followed the usual logarithmic pattern. Strophanthin, and especially quinidine, caused delayed recovery and more prolonged refractoriness of both regions of the heart. Strophanthin prolonged the Q-T interval in small doses and usually shortened it in larger doses. Quinidine effects upon the Q-T interval were unexpectedly variable, contrary to the usual prolongation in thyroton driven ventricles and in clinical cases.

¹¹ Decherd, G. M., and Ruskin, A., *Brit. Heart J.*, 1946, **8**, 6.

¹² Ruskin, A., and Decherd, G. M., unpublished data.

16518

Hemochromogens and Related Compounds in Liquid Ammonia.*

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A study of the reactions and reaction products of hemin and coordinating substances in liquid ammonia by means of spectroscopy and bio-assay has not been made previously.

However, the senior author¹⁻⁵ has made various studies on the reactions of ammonolyzed and ammonated proteins, protein derivatives and hemin. Some of the reactions in liquid

* The authors wish to thank Armour and Company and the Wellcome Research Laboratories for their generous gifts of dried blood proteins, and the Illinois Department of Public Health for the supplies of desiccated plasma and liquid globulin.

¹ Roberts, R. G., and Miller, C. O., *Proc. Soc. Exp. Biol. and Med.*, 1933, **30**, 821.

² Roberts, R. G., and Miller, C. O., *Proc. Soc. Exp. Biol. and Med.*, 1934, **31**, 522.

³ Roberts, R. G., Tweedy, W. R., and Smullen, C. H., *J. Biol. Chem.*, 1935, **112**, 209.

⁴ Roberts, R. G., and Miller, C. O., *J. Am. Chem. Soc.*, 1936, **58**, 309.

⁵ Roberts, R. G., *J. Biol. Chem.*, 1939, **128**, 597.

ammonia or the nitrogen system, which are to be described below, will be compared to similar ones in water or the oxygen system. Franklin⁶ first demonstrated the value of comparing certain substances in regard to their classifications in the nitrogen and oxygen systems. He did not work with the hemochromogens, however.

There are certain marked differences in the behavior of hemin in the two solvents, some of which should be mentioned at this point. Hemin is soluble in liquid ammonia but reacts with it to form conjugates so that the ammonia itself is a coordinating substance. This does not exclude the formation of other hemochromogens or coordinates, however, as will be shown later. Hemin is practically insoluble in water at the neutral point or pH 7.0 but is soluble in aqueous alkali at pH 8.0 to 9.0. A search of the chemical literature has failed to show that the pH of liquid ammonia has been determined experimentally. The ionization constant of liquid ammonia, however, as calculated from its electrical conductivity is 1.9×10^{-33} . As stated by Plexkov and Monoszon⁷ pure liquid ammonia ionizes as follows: $\text{NH}_3 \rightarrow \text{H}^+ + \text{NH}_2^-$ or $2\text{NH}_3 \rightarrow \text{NH}_4^+ + \text{NH}_2^-$ and $(\text{NH}_4^+) (\text{NH}_2^-) = 1.9 \times 10^{-33}$ at -50°C . The NH_4^+ ion is in greater abundance than the H^+ ion. Lykken⁸ recommends that the term pH should be reserved for aqueous systems only, and that the term cG should be used for non-aqueous systems.

Experimental. The liquid ammonia used at the beginning of this work was dried over sodium by the method of Fernelius and Johnson.⁹ It was found later, however, that for bio-assay or spectroscopy the conjugates or coordinates which were prepared in the anhydrous liquid ammonia made by Mathieson or du Pont could not be distinguished from the coordinates prepared in liquid ammonia

which had been dried over sodium. The laborious and time consuming drying operation was, therefore, discontinued. The addition of the reactants to the liquid ammonia and the isolation of the reaction products have been described in previous publications.

A Gaertner high dispersion spectroscope was used for the spectroscopic studies of those compounds and coordinates that remained dissolved or dispersed in liquid ammonia. The liquid ammonia and solutes were contained in a small Dewar flask that was mounted directly in front of the entrance slit of the spectroscope. The vacuum flask was connected to a mercury seal by rubber tubing to permit the ammonia gas to escape and the entire apparatus was placed in a well ventilated hood. Spectrophotometric determinations were made with a Beckman quartz spectrophotometer upon ammonolyzed and/or ammonated compounds or conjugates that had been dissolved or dispersed in water, ethylene glycol, or other media with the exception of liquid ammonia itself. Franklin used the term ammono to designate ammonolyzed compounds, which in some cases were also ammonated. The term ammano as used in this paper is a more general one and means ammonia treated. Such a term is necessary especially when proteins are being considered since one often does not know the exact nitrogen distribution.

Discussion. When liquid ammonia reacts with an iron porphyrin, it is acting as a coordinating substance in the same general way that nitrogen bases act as coordinating substances to produce hemochromogens. Several of these coordinating substances (No. 1 to 16), among which is galactose, are shown in Table I. That ammano-iron porphyrin No. 1, red in liquid ammonia and absorbing at $555 \text{ m}\mu$, might be a complex formed by ammonolysis and ammonation when hemin chloride is treated with an excess of liquid ammonia, is indicated by Kjeldahl analysis. Using 610.95 g as the gram molecular weight of hemin chloride, we found that there is an increase of 19.39 g of nitrogen per g.m.w. after the sample has remained for 24 hours over sulfuric acid in a vacuum desiccator at $22-24^\circ\text{C}$, but only 13.6 g under vacuo at $90-$

⁶ Franklin, *The Nitrogen System of Compounds*, Reinhold Pub. Corp., 1935.

⁷ Plexkov and Monoszon, *Acta Physicochim. U.S.S.R.*, 1935, **1**, 725.

⁸ Lykken, L., Symposium on pH Measurement, Am. Soc. Test. Mat., 1946, Tech. Pub. No. 73.

⁹ Fernelius, W. C., and Johnson, W. C., *J. Chem. Ed.*, 1929, **6**, 444.

TABLE I.
Hemochromogens Formed in Liquid Ammonia and Dispersed in Various Solvents.

No.	Coordinating Substance	Solvent	Absorption areas and curves Wave length, $m\mu$	Reaction products and related data.
1.	Liquid NH_3	Liquid NH_3	Band 555	Brownish-black powder soluble in H_2O at pH 5.5, and in ethylene glycol.
2.	Liquid NH_3	Water	Broad shallow curve. Center 623.	Similar to hematin albumin absorption found in plasma of malaria patients.
3.	Pyridine	Liquid NH_3	Band at 540	Band at 555 disappears immediately on addition of pyridine.
4.	Ammano-pyridine	Water	Broad deep curve. Center at 540.	Failure of band at 540 to shift on change of solvent is unusual.
5.	Galactose	Liquid NH_3	Diffuse band 567-521. Sharp area 559.	Brownish-black powder. Forms red sol. in liquid NH_3 similar to ammano-iron porphyrin.
6.	Ammano-galactose	Water	Shallow curve.	On changing from liquid NH_3 to water the band narrows and shifts toward the red spectrum.
7.	Liquid NH_3	Ethylene glycol	Center at 640. Broad deep curve. Center at 600.	Ammano-iron porphyrin very soluble in ethylene glycol. Iron porphyrin is not soluble.
8.	Liquid NH_3	Ethylene glycol plus guanidine thiocyanate	Broad deep curve. Center at 500.	A small amt. of guanidine thiocyanate causes shift of 100 $m\mu$.
9.	Caffeine	Liquid NH_3	Bands α 577, β at 548.	Red powder turning to brown on exposure to atmosphere.
10.	Ammano-caffeine	Water	Wide curve. Center at 576.	Caffeine porphyrin absorbs at 570 and 540 in water.
11.	Ammano-histidine	Water	General curve. No specific absorption.	Absence of specific absorption indicates reaction between histidine and iron porphyrin.
12.	Ammano-nicotinic acid	Water	General curve. Identical with ammano-histidine.	Identity of curves of ammano-histidine and ammano-nicotinic acid indicates similar properties.
13.	Ammano-adrenalin	Water	Shallow curve. Center at 540.	Absence of specific absorption indicates coordination of iron porphyrin with adrenalin.
14.	Ammano-choline chloride	Ethylene glycol	Deep curve. Center at 260.	Choline chloride absorbs at 260. The difference in intensity indicates a reaction.
15.	Ammano-insulin	Ethylene glycol	No specific absorptions. (Fig. 1.)	Disappearance of both choline chloride and insulin absorption indicates a reaction.
16.	Ammano-choline chloride	Aqueous sol. of dried beef plasma	Curve centering at 532 and 565. (Fig. 2.)	Red powder absorptions lie midway between those obtained for haem albumin and serum hemochromogen.

100°C, and only 6.05 g after washing with water and drying at 101°C. The last increase is approximately one-half of a gram atom of nitrogen and might indicate the existence of an ammonia bridge between 2 iron porphyrin molecules.

Ammano-iron porphyrin No. 2 is soluble in water below pH 7.0, forming a brownish-amber solution which absorbs between 605-635 $m\mu$. This absorption band resembles that of a cross between the absorption band of acid haematin described by Newcomer¹⁰ and the absorption band of free alkaline haematin as given by Heilmeyer.¹¹ The latter also reported a band of similar shape centering at 623 $m\mu$ for a blood pigment obtained from clinical cases of "haematin jaundice." Any clinical comparison of the above mentioned bands is of academic interest only, at the present time.

The ammano-pyridine iron porphyrins No. 3 and 4, prepared according to the directions given by Drabkin and Austin,¹² absorbs at 540 $m\mu$ in either liquid ammonia or water. Ammano-galactose iron porphyrin No. 5, prepared by using equal weights of galactose and hemin, absorbs in a very broad band between 567 and 521 $m\mu$ with a sharp peak at 539 $m\mu$. This range of absorption would include the class of substances named hemochromogens, such as serum hemochromogen which absorbs with the α band at 558 $m\mu$ and the β at 528 $m\mu$ (α represents absorption in the longer wave lengths of a series of bands and β in the shorter wave lengths, etc.). There is also the class of substances named haem-albumins by Fairley¹³ and Keilin,¹⁴ such as serum haem-albumins, α at 570 $m\mu$ and β at 540 $m\mu$. In water, ammano-galactose iron porphyrin No. 6 absorbs at 640 $m\mu$. Keilin states that only caffeine reacts with hemin to give products absorbing in the range of haem-albumins. Ammano-caffeine iron porphyrin

No. 9, prepared according to the directions given by Keilin, absorbs at α 577 $m\mu$ and β at 548 $m\mu$ in liquid ammonia. In water ammano-caffeine iron porphyrin No. 10 gives a wide curve centering at 576 $m\mu$.

An interesting theory in regard to the important role possibly played by histidine in the formation of cross linkages between protein molecules and other molecules as hemin in such compounds as hemoglobin and cytochromes has been proposed by Keilin.¹⁵ For a comparison, histidine was allowed to react with hemin chloride in liquid ammonia and the reaction product, ammano-histidine iron porphyrin No. 11, prepared according to the directions of Keilin, was dissolved in water and studied spectrophotometrically. The absence of any specific absorption indicates that a reaction probably occurred, since the individual reactants have specific absorptions. However, nicotinic acid No. 12, prepared in a similar way to the histidine product, with proper consideration of equivalent weights, reacted in a similar way, and gave almost identical general absorption curves. The reaction of histidine in this regard is, therefore, not unique. Adrenalin No. 13, prepared in a similar manner, acts in the same way spectrophotometrically as nicotinic acid and histidine. Adrenalin conjugates give a prolongation of elevated blood pressure in dogs.

Ammano-insulin choline chloride iron porphyrin No. 15 prepared by using 20 mg of insulin, 20 mg of hemin, and 40 mg of choline chloride, is soluble in ethylene glycol but gives no specific absorption (Fig. 1), although ammano-choline chloride iron porphyrin No. 14, absorbs strongly at 250 $m\mu$ and insulin, either crystalline or amorphous, absorbs strongly at 275 $m\mu$ as has been shown by Roffo.¹⁶ When ammano-insulin choline chloride iron porphyrin dispersed in ethylene glycol is injected subcutaneously into rabbits it gives a prolongation of lowered blood glucose lasting 5 times as long as that of insulin controls.

¹⁰ Newcomer, H. S., *J. Biol. Chem.*, 1919, **37**, 465.

¹¹ Heilmeyer, L., *Deutsch. Arch. Klin. Med.*, 1932, **173**, 128.

¹² Drabkin, D. L., and Austin, J. H., *J. Biol. Chem.*, 1935-36, **112**, 51.

¹³ Fairley, N. H., *Brit. J. Exp. Path.*, 1940, **21**, 231.

¹⁴ Keilin, J., *Nature*, 1944, **154**, 120.

¹⁵ Keilin, J., *Biochem. J.*, 1943, **37**, 281.

¹⁶ Roffo, A. N., and Francone, M. P., *Boletin Instituto de Medicina Experimental*, 1941, **18**, 1003.

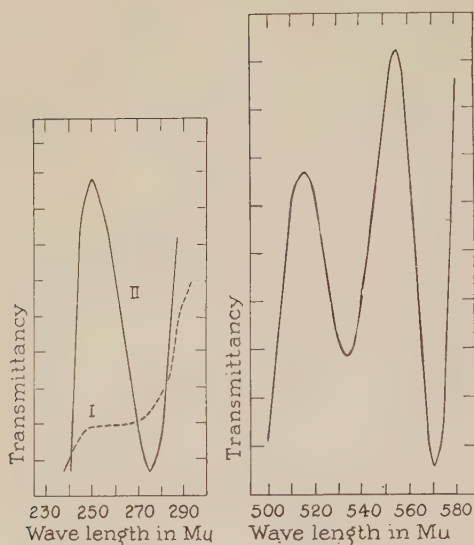


FIG. 1. (left)

Curve I, the absorption curve of Ammano-insulin choline chloride iron porphyrin. Curve II, the absorption curve of plain insulin. The thickness of the absorbing liquid was 1.0 cm in both cases. The concentration of the coordinated iron porphyrin as shown in curve I was 0.2 g in 100 cc in terms of the insulin content. The concentration of plain insulin as shown in curve II was 0.2 g in 100 cc and the transmittancy was plotted between zero and 100% for both curves.

The smoothed curves were readings on the Beckman spectrophotometer, at 5 $m\mu$ intervals, and at inflection points at even less than 5 $m\mu$. If all these points were plotted, they would almost touch each other.

FIG. 2. (right)

The absorption curve of Ammano-egg albumin iron porphyrin. The thickness of the absorbing liquid was 1.0 cm. The concentration of the coordinated iron porphyrin was 0.1 g in 100 cc and the transmittancy was plotted between zero and 100%.

Both Fairley and Keilin state that haem-albumin can be obtained only when human serum is used to conjugate with the haem. However, ammano-egg albumin iron porphyrin absorbs at α 565 $m\mu$ and β at 532 $m\mu$ (Fig. 2). Since these wave lengths are somewhat short for haem-albumins and somewhat long for hemochromogens, the ammano-conjugate or coordinate might be considered as an ortho haem-albumin or as a meso hemochromogen, and in all likelihood represents a new class of porphyrins—the ammano-iron porphyrins.

Several other proteins form red reaction products with hemin in liquid ammonia and

they probably belong in the same class of ammano derivatives as ammano-egg albumin iron porphyrin. As a rule it is difficult to get ammano protein iron porphyrins into solution and they usually lack characteristic specific absorptions. Among them are bovine beta globulin, bovine gamma globulin, castor bean globulin, bovine fibrinogen, fibrin foam, bovine albumin and human albumin. The red reaction products form as readily with the globulins and fibrins as with the albumins. Globin also forms a red reaction product and when globin and hemin are mixed in the same proportion in which they occur in hemoglobin, an α band occurs at 580 $m\mu$ (α hemoglobin 578 $m\mu$) a β band at 528 $m\mu$ (β hemoglobin 540 $m\mu$) and a gamma band at 465 $m\mu$ which is not found at all in hemoglobin.

Summary. 1. A study of the reactions and reaction products of hemin and coordinating substances and certain compounds and conjugates related to them has been made in liquid ammonia.

2. The nomenclature used to describe new compounds and conjugates that are formed in liquid ammonia has been adopted primarily from the nomenclature as used by Keilin for derivatives of hemin and from the nomenclature as used by Franklin in his studies of the nitrogen system of compounds.

3. A new derivative of hemin and liquid ammonia has been described, which exists only in liquid ammonia and which absorbs strongly at 555 $m\mu$.

4. A compound has been prepared by dispersing liquid ammonia treated hemin in water. This compound absorbs at 620 $m\mu$, which is the same spectral region as that found for some hemin compounds occurring in the blood of patients suffering from black water fever. The ammano-hemin is soluble in water at pH 5.5 and it is also soluble in ethylene glycol.

5. A new preparation has been made by combining egg albumin with hemin in liquid ammonia. This conjugate absorbs in two bands at 532 $m\mu$ and 565 $m\mu$. These bands lie midway between those obtained by other workers for haem-albumin and serum hemochromogen. Fairley and Keilin were able to

prepare haem-albumin only when serum albumin was used.

6. Caffeine hemin can be prepared in liquid ammonia. It absorbs at $577\text{ m}\mu$ and $548\text{ m}\mu$ when it is dispersed in liquid ammonia.

7. Insulin reacts with choline chloride and

hemin in liquid ammonia to form a conjugate in which the strong absorption band of insulin at $275\text{ m}\mu$ is missing, yet this conjugate maintains a prolongation of lowered blood glucose of 5 times the duration obtained by insulin controls.

16519

Determination of Para-Aminosalicylic Acid in Blood.*

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Since para-aminosalicylic acid has been found to have a definite chemotherapeutic effect in experimental tuberculosis, a simple method for its determination in blood appears desirable. The sulfonamide method in general use¹ cannot be employed without modification. In view of several requests for a method for determination of this substance, and since the slight modification of the sulfonamide method found necessary may prove useful with other aryl amines, we are publishing this short note.

No color is obtained when a solution of p-aminosalicylic acid is subjected to the ordinary procedure for determining sulfonamides. If the acidity of the solution is increased a color is obtained. Maximum color is developed at room temperature when the solution is made about 3 N with hydrochloric acid. The results are, however, not consistent. Diazotization for one minute gives a more intense color than for 3 or 10 minutes. Obviously, either the diazo compound is unstable or a secondary reaction is occurring with the nitrous acid. Diazotization done with the solution at 1°C and made 1 N with hydrochloric acid yielded a more intense color than any other procedure which we have tried. The results under these conditions appear to be

quite consistent and reproducible.

Reagents. 1. A solution of trichloroacetic acid containing 15 g dissolved in water and diluted to 100 cc.

2. A 0.1% solution of sodium nitrite.

3. An aqueous solution of N-(1-naphthyl)-ethylenediamine dihydrochloride containing 100 mg per 100 cc. This solution should be kept in a dark colored bottle.

4. 6 N hydrochloric acid.

5. A solution of ammonium sulfamate, containing 0.5 g per 100 cc.

6. A standard solution of p-aminosalicylic acid prepared by suspending 50 mg of the compound in water and dissolving in slightly more than the theoretical amount of sodium hydroxide, and diluting to one liter. Dilutions are made from this stock solution in order to prepare a calibration curve for the colorimeter.

Procedure. Blood or plasma is diluted 1:50 or, if very low values are expected, 1:20. For 1:50 dilution, use 1 part of blood or plasma, 39 parts of water and 10 parts of trichloroacetic acid solution. To 10 cc of the clear filtrate are added 2 cc of 6 N hydrochloric acid, and then the solution is cooled in ice (to about 1°C). One cc of the solution of sodium nitrite is added and after 3 minutes, 1 cc of the solution of sulfamate. The tubes are removed from the ice bath, and after 2 minutes, 1 cc of the solution of N-(1-naphthyl)ethylenediamine dihydrochloride is added. Read-

* This investigation has been aided by a grant from the U. S. Public Health Service.

¹ Bratton, A. C., and Marshall, E. K., Jr., *J. Biol. Chem.*, 1939, **128**, 537.

ings are taken with a photoelectric colorimeter after the solutions have stood for 20 minutes in the dark. The zero is set with 10 cc of 3% trichloroacetic acid to which the reagents have been added (6 N hydrochloric acid, nitrite, sulfamate and coupler).

Recovery. For a 1:50 dilution of whole

blood, the average recovery of added p-aminosalicylic acid was 90% (86-95); for a 1:50 dilution of plasma, the average recovery was 95% (91-97); and for a 1:20 dilution of plasma, the average recovery was 93% (89-96).

16520

Distribution of 3,4-Dimethyl-5-sulfanilamidoisoxazole in the Body.*

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Sulfanilamide has been found to be distributed in all tissues and fluids of the body in approximately equal concentration.¹ Since the apparent volume of distribution of sulfanilamide is 87 to 98% of the body weight, which is somewhat in excess of the water content of the body (about 65%), it must be localized to a slight degree in tissue.² Sulfapyridine is distributed in a manner similar to that of sulfanilamide.^{3,4} Sulfathiazole, sulfadiazine, sulfapyrazine and sulfamerazine are distributed in a volume corresponding to 45 to 55% of the body weight,⁴ indicating that they penetrate tissues but to a lesser extent.[†]

* I wish to thank Dr. C. Gordon Zubrod for aid in the studies on patients and Dr. Elmer L. Sevringhaus of Hoffmann-LaRoche, Inc., for supplying the drug used in this study.

¹ Marshall, E. K., Jr., Emerson, K., Jr., and Cutting, W. C., *J. Pharm. and Exp. Therap.*, 1937, **61**, 196.

² Waterhouse, A., and Shannon, J. A., *PROC. SOC. EXP. BIOL. AND MED.*, 1942, **50**, 189.

³ Marshall, E. K., Jr., and Litchfield, J. T., Jr., *J. Pharm. and Exp. Therap.*, 1939, **67**, 454.

⁴ Fisher, S. H., Troast, L., Waterhouse, A., and Shannon, J. A., *J. Pharm. and Exp. Therap.*, 1943, **79**, 373.

† In addition to data given in the literature for distribution of these drugs, I have unpublished experiments showing that in the dog sulfanilamide is distributed in 87% and sulfadiazine and sulfapyrazine in 48% of the body weight.

The data to be presented indicate that 3,4-dimethyl-5-sulfanilamidoisoxazole is distributed differently from any of the sulfonamides in clinical use; it appears to be confined to the extracellular water, with a volume of distribution of the order of magnitude of 25-30% of the body weight.

Methods. Determinations of the distribution of the drug were done on 3 dogs and 4 patients. The drug was injected intravenously; in the dog the sodium salt was used and in man, the lithium salt. About 50 mg per kilo were given in each case. Blood samples were taken at 0.5, 1, 1.5, 2 and 2.5 hours and urine was collected for the first hour after injection. The drug was determined in plasma and urine by the usual sulfonamide method.⁵ In man both free and total sulfonamide were estimated; in the dog, no conjugated drug was found. The amount of conjugated drug in either the plasma or urine taken at 1 hour was 3% or less in 3 of the individuals and was about 10% in the other. All calculations have been based on the figures for free sulfonamide. No great error is involved.

Results. The apparent volume of distribution in per cent of body weight was calculated from the data obtained in two ways. The logarithms of plasma concentrations were

⁵ Bratton, A. C., and Marshall, E. K., Jr., *J. Biol. Chem.*, 1939, **128**, 537.

TABLE I.
Apparent Volume of Distribution of 3,4-dimethyl-5-sulfanilamidoisoxazole in % of body weight.

Dog	Calculated from	
	Zero time	1 hr
1	33	
2	26	30
3	33	29
Patient		
H.W.	25	24
L.H.	24	23
G.B.	28	29
C	22	22

plotted against time, and the resulting straight line extrapolated to zero time. The value obtained, together with the dose given per kg, allowed a calculation of the distribution in per cent of body weight. The other method used was to calculate the distribution from the concentration in the plasma at 1 hour assuming that the amount of drug excreted in the urine during this hour represents the total amount disappearing from the body in that time. The results of these calculations are given in Table I.

Discussion. 3,4 dimethyl-5-sulfanilamidoisoxazole has been studied experimentally by Schnitzer *et al.*⁶ Both the drug and its acetyl derivative are much more soluble in water than the sulfonamides in clinical use; in this respect, it resembles sulfanilamide. One would,

therefore not anticipate any renal toxicity due to precipitation of the drug in the urinary tract. It is also stated to be quite effective against bacteria susceptible to the sulfonamides. The drug has been used to a limited extent clinically.⁷⁻¹⁰ The observation that the drug is apparently distributed in extracellular water only has two important implications for its clinical use. These are 1) the same amount of drug in the body will give a concentration in the plasma 3 times that for sulfanilamide and about twice that for sulfadiazine and sulfamerazine, and 2) its apparent failure to penetrate cells should make it less toxic than sulfonamides which readily enter the tissues.

Summary. The distribution of 3,4 dimethyl-5-sulfanilamidoisoxazole has been studied in the dog and in man. Its apparent volume of distribution in per cent of the body weight indicates that it is contained only in extracellular water.

⁶ Schnitzer, R. J., Foster, R. H. K., Ercoli, N., Soo-Hoo, G., Mangieri, C. N., and Roe, M. D., *J. Pharm. and Exp. Therap.*, 1946, **88**, 47.

⁷ Sarnoff, S. J., Freedman, M. A., and Hyman, A. A., *J. Urol.*, 1946, **55**, 417.

⁸ Haines, W. H., and Micali, S., *Pennsylvania Med. J.*, 1947, **50**, 1328.

⁹ Narins, L., *J. Urol.*, 1948, **59**, 92.

¹⁰ Rodgers, R. S., and Colby, F. H., *J. Urol.*, 1948, **59**, 659.

16521 P

Quantitative Study of Effect of Antrum Resection on Gastric Secretion in Pavlov Pouch Dogs.*

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In 1906, Edkins¹ found that extracts of antral mucosa stimulated gastric secretion when administered intravenously. Three years later, Edkins and Tweedy² reported that secre-

tion of acid by the fundus could be produced by introducing various food substances into the antrum separated from the fundus by a diaphragm. Edkins postulated from these studies that this fundic secretion was due to a hormone, which he named gastrin, and which was produced by the antral mucosa in

* This work was aided by grants from the Douglas Smith Foundation for Medical Research, the Kenneth G. Smith Medical Research Fund, and Mr. Andrew E. Wigeland.

¹ Edkins, J. S., *J. Physiol.*, 1906, **34**, 133.

² Edkins, J. S., and Tweedy, J. M., *J. Physiol.*, 1909, **38**, 263.

TABLE I.
Effect of Antrum Resection on Average 24 hr Secretion of Pavlov Pouch Dogs.

Dog No.	Before resection				After resection			
	No. of days	Vol. in cc	Free acid in clinical units	HCl in m.eq.	No. of days	Vol. in cc	Free acid in clinical units	HCl in m.eq.
D-928	17	733	120	83.8	21	246	70	17.4
D-939	25	539	124	67.1	29	79	7	0.6
D-944	16	989	122	122.7	20	439	99	43.7
D-879	29	863	129	112.0	24	157	36	6.1

response to chemical stimulation by food stuffs.

Ivy and Whitlow,³ using Pavlov pouch dogs with an isolated antral pouch, obtained no acid secretion after stimulation of the antral mucosa with food substances. Therefore, Ivy concluded from this and other studies⁴ that the substance causing the chemical or hormonal phase of gastric secretion was for the most part elaborated by the upper small intestine. Smidt,⁵ working with Pavlov pouch dogs, reported marked reduction in the secretion of acid after antrum resection. Portis and Portis,⁶ Shapiro and Berg,⁷ and Thompson,⁸ found little or no change in acid secretion in Pavlov pouch dogs after antrectomy.

For the most part, all of the above work was qualitative rather than quantitative, the

conclusions being based upon the concentration of free acid in fractional samples. For this reason, it was decided to re-investigate the importance of the antrum in the chemical phase of gastric secretion using the quantitative methods of Dragstedt, Haymond, and Ellis.⁹

In 4 dogs, large Pavlov pouches were constructed which were drained by gold-plated brass cannulas. The 24 hour pouch secretion was collected in a detachable rubber bladder, and volume, free acidity, and hydrochloric acid content determined. The animals were fed a standard diet supplemented by oral sodium chloride. After a period of observation, the entire antrum was resected, and following recovery, comparable data collected.

Results and conclusions. In all animals, antrum resection was followed by a pronounced reduction in pouch secretion (Table I). The average reduction in the 24 volume was 73%, and in free acidity, 57%. The reductions in average 24 hour hydrochloric acid output were 79, 99, 64, and 95% respectively, averaging 84%. These data indicate that the antrum is important in the chemical or hormonal phase of gastric secretion, and supports Edkins' hypothesis.

³ Ivy, A. C., and Whitlow, J. E., *Am. J. Physiol.*, 1922, **60**, 578.

⁴ Lim, R. K. S., Ivy, A. C., and McCarthy, J. E., *Quart. J. Exp. Physiol.*, 1925, **15**, 13.

⁵ Smidt, H., *Arch. v. Klin. Chirurg.*, 1923, **125**, 26.

⁶ Portis, S., and Portis, B., *J. A. M. A.*, 1926, **86**, 836.

⁷ Shapiro, P. F., and Berg, B. N., *Proc. Soc. Exp. Biol. and Med.*, 1932, **39**, 743.

⁸ Thompson, H. L., *Calif. and West. Med.*, 1932, **36**, No. 6.

⁹ Dragstedt, L. R., Haymond, H. E., and Ellis, J. C., *S. G. O.*, 1933, **56**, 799.

16522 P

Observations on Effects of Altitude on Renal Function.

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(Introduced by A. W. Hetherington.)

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At the present time there is a paucity of data available on the effect of reduced atmospheric pressure on the excretory function of the kidney. Alving *et al.*¹ studied the effects on renal function of intermittent, daily exposure of young men to simulated altitudes of 10,000 to 18,000 feet. Observations were made from the thirteenth to the forty-first day of exposure. They found that such exposure caused no change in glomerular filtration rate, but did cause an increase in maximum tubular excretory ability for diodrast (TmD).

We have been unable to find any specific data concerning the effect of simple acute hypoxia on unanesthetized experimental subjects as regards glomerular filtration rate, maximum tubular excretory ability; nor maximum tubular reabsorptive capacity. It is the influence of simple acute hypoxia on these phenomena with which this report is concerned.

Methods. Five well trained, unanesthetized, adult female dogs, 2 pure-bred Dalmatian coach hounds and 3 mongrels, were employed in these studies. The urine collection periods ranged from 10 to 15 minutes each with all standard precautions taken to insure adequate urine flow and bladder evacuation. Blood samples were obtained by external jugular puncture, and heparin was used as an anticoagulant. The renal function tests were performed at ground level and in an altitude

chamber at simulated altitudes of 18,000 and 24,000 feet.

The glomerular filtration rate (GF) was measured by the clearance of creatinine (C_{cr}) in the mongrel dogs and by the clearance of inulin (C_{in}) in the Dalmatians. The maximum tubular excretory ability was measured with para-aminohippuric acid (TmPAH), and the maximum tubular reabsorptive capacity was measured with glucose (TmG). Inulin and para-aminohippuric acid were determined by the methods outlined by Smith *et al.*² creatinine by the method of Folin and Wu,³ and glucose by the Folin method.⁴

The plasma levels of the various test substances were maintained following the injection of a primer dose by constant intravenous infusions started at least 20 minutes previous to the first urine collection period. Several consecutive urine collection periods were studied each day. To minimize the possible effects of acclimatization no animal was submitted to exposure to altitude more frequently than once per week. Because of the reported mutual interference of simultaneous TmPAH and TmG determinations,^{5,6} only one of these measurements was performed on any one animal during a single exposure to altitude.

Results. The results obtained are summarized in Tables I and II. Although fluctuations in glomerular filtration rate were ob-

* Present address: Dr. Kelley, Dept. of Pediatrics, Univ. of Minn., Minneapolis; Dr. McDonald, Section on Gerontology, National Institute of Health, Bethesda, Md.

¹ Alving, A. S., Adams, W., Bay, E. B., Bryan, A. H., Carmichael, H. T., Case, T., Halstead, W. C., Landowne, M., and Ricketts, H. C., Studies of Renal Function, CAM Report No. 135, June 16, 1943.

² Smith, H. W., Finkelstein, N., Alminosa, L., Crawford, B., and Graber, M., *J. Clin. Invest.*, 1945, **24**, 388.

³ Folin, O., and Wu, H., *J. Biol. Chem.*, 1919, **38**, 81.

⁴ Folin, O., *J. Biol. Chem.*, 1929, **82**, 83.

⁵ Houck, C. R., *Proc. Soc. Exp. Biol. and Med.*, 1946, **63**, 398.

⁶ Klopp, C., Young, N. F., and Taylor, H. C., Jr., *J. Clin. Invest.*, 1945, **24**, 117.

TABLE I.
Effect of Altitude on TmPAH.

		Ground level			18,000 feet			24,000 feet		
Dog		Date	GF* cc/min	TmPAH† mg/min	Date	GF cc/min	TmPAH mg/min	Date	GF cc/min	TmPAH mg/min
No. 1	Wt 8.6 kg	3-11-47	55.0 ¹⁰	9.1 ⁵	3-18-47	43.8 ⁸	13.1 ⁴	4-11-47	67.8 ⁸	8.2 ⁴
No. 2	Wt 10.5 kg	20	44.9 ⁹	22.6 ⁴	27	47.7 ⁷	15.3 ⁴	4-8	43.3 ⁸	25.8 ⁴
		9-24	53.2 ³		9-24	58.1 ⁴		9-24	53.8 ⁴	
		10-13	39.7 ⁷	27.0 ⁷						
		23	46.1 ⁷							
No. 3	Wt 8.6 kg	3-7	41.8 ⁸	20.1 ⁴	3-14	98.5 ¹⁰	37.7 ⁵	4-7	58.6 ⁹	65.1 ⁴
					4-24	58.6 ⁴				
No. 4	Wt 21.8 kg	4-21	105.9 ⁵		9-15	64.2 ⁴	88.9 ⁴	9-15	63.6 ³	204.3 ³
		5-5	97.4 ⁵							
		9-15	95.5 ⁴	87.0 ⁴						
		10-16	87.3 ⁴							
		11-3	100.8 ⁴							
No. 5	Wt 22.7 kg	5-19	99.8 ¹⁰		23	88.0 ⁴	83.2 ⁴	23	61.0 ⁴	78.7 ⁴
		8-5	110.0 ⁵							
		9-18	96.0 ³	85.6 ³						
		23	95.0 ⁴	75.5 ⁴						
		10-31	94.9 ⁹							
		11-6	88.0 ³							

Superscripts refer to the number of periods for which the figure quoted is the mean value.

* Glomerular filtration rate.

† Maximum tubular excretory ability for para-aminohippuric acid.

TABLE II.
Effect of Altitude on TmG.

		Ground level		18,000 feet		24,000 feet	
Dog	Date	GF* cc/min	TmG† mg/min	GF cc/min	TmG mg/min	GF cc/min	TmG mg/min
No. 2	Wt 10.5 kg	10-1-47	42.8 ⁴	157.9 ⁴	44.3 ⁴	126.6 ⁴	46.4 ⁴
		9	48.1 ⁴	134.9 ⁴	42.8 ⁴	137.5 ⁴	143.7 ⁴
No. 4	Wt 21.8 kg	9-29	94.9 ⁴	352.3 ⁴	88.5 ⁴	164.7 ⁴	106.2 ⁴
No. 5	Wt 22.7 kg	10-8	85.2 ³	394.3 ³	86.0 ³	316.0 ³	92.5 ⁴

Superscripts indicate the number of periods for which the figure quoted is the mean value.

* Glomerular filtration rate.

† Maximum tubular reabsorptive capacity for glucose.

served, no obvious trend is exhibited by these changes. It is felt that all are attributable to individual variability of response to altitude. In 4 of the dogs at 18,000 feet and in 3 of them at 24,000 feet, no significant changes in TmPAH occurred. In dog No. 3 at 18,000 feet and in dogs Nos. 3 and 4 at 24,000 feet, significant increases in the TmPAH were observed. These changes were 88%, 222%, and 135%, respectively.

TmG was somewhat decreased by exposure to altitude. At 18,000 feet there was in 1 animal a relatively small decrease of approxi-

mately 20%, in another a larger decrease of approximately 55%, and in another no change in TmG in two sets of experiments. Although there were quite marked changes in TmG at 18,000 feet as compared to ground level, there were no appreciable differences observed between the values obtained at 18,000 and at 24,000 feet.

Discussion. Theoretically, decreased TmG and TmPAH could result from decreased availability of oxygen to the kidney tubules. In the present studies TmG did decrease at altitude but TmPAH did not. Indeed, there

is an apparent increase in some cases in the ability of the tubule cells to excrete PAH upon exposure of the animal to an atmosphere of sufficiently reduced oxygen tension. This observation agrees with that of Alving *et al.*¹ in the case of chronic, intermittent exposure to anoxia.

⁷ Eiler, J. J., Althausen, T. L., and Stockholm, M., *Am. J. Physiol.*, 1943, **140**, 699.

⁸ Heinbecker, P., Rolf, D., and White, H. L., *Am. J. Physiol.*, 1943, **139**, 543.

⁹ Welsh, C. A., Rosenthal, A., Duncan, M. T., and Taylor, H. C., Jr., *Am. J. Physiol.*, 1942, **137**, 338.

An increase in the maximum tubular excretory ability has been effected by the administration of various hormone preparations,⁷⁻⁹ indicating that hormonal influences are capable of causing alterations in the tubular transfer mechanism of the kidney. The possibility of such influences being operative here must be considered.

Conclusions. Upon exposure of animals to altitude, 1) glomerular filtration rate response varies widely with the individual, 2) TmPAH is increased or unaffected, and 3) TmG is decreased or unaffected.

16523

On Phenyletherase.*

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The enzymatic cleavage of the carbon-oxygen bond of phenyl ethers by rat tissues is reported in this paper. The methoxy-phenyl linkage occurs in a variety of biologically active compounds which include anti-fibrillatory compounds such as quinine, quinidine and α -fagarine¹ although here the methoxy radical apparently is not necessary² for the physiologic activity. This linkage also occurs in the anti-malarial compounds, atabrine and pentaquine; in the sedative papaverine; and in certain estrogen compounds such as dimethoxystilbestrol. The methoxy-phenyl linkage is present in the local anesthetic phenacaine. Barry, O'Rourke and Twomey³ have shown that diphenyl ethers have potent anti-tuberculous activity *in vitro*.

* This work was supported by grants from the American Cancer Society recommended by the Committee on Growth of the National Research Council and from Mr. Ben May, Mobile, Alabama.

¹ Moisset de Espanés, E., and Weksler, B., *Proc. Soc. Exp. Biol. and Med.*, 1946, **63**, 195.

² Di Palma, J. R., and Lambert, J. J., *Science*, 1948, **107**, 66.

³ Barry, V. C., O'Rourke, L., and Twomey, D., *Nature*, 1947, **160**, 800.

We have been unable to find earlier reports on enzymatic decomposition of ethers. The enzyme pectase catalyzes the cleavage of "methoxyl" groups of soluble pectin, a reaction studied extensively by Kertesz.^{4,5} However, in pectin the methyl groups are attached to the carbohydrate as an ester and this bond is considerably weaker than an ether linkage. *p*-Nitrophenyl esters⁶ are attacked at a much faster rate by esterase than are its ethers by the enzyme under consideration here.

It was found that the splitting of the methyl and ethyl ethers of *p*-nitrophenol (PNP) is carried out extensively by the intact rat and slightly by homogenates of liver and kidney. Following injection of these compounds in rats, appreciable or even large amounts of free and combined *p*-nitrophenol are excreted in the urine; it is well known that phenols are in large part excreted in the urine as conjugates of glucuronic and sulfuric acids. Since *p*-

⁴ Kertesz, Z. I., *J. Biol. Chem.*, 1937, **121**, 589.

⁵ McColloch, R. J., and Kertesz, Z. I., *J. Biol. Chem.*, 1945, **160**, 149.

⁶ Huggins, C., and Lapides, J., *J. Biol. Chem.*, 1947, **170**, 467.

nitrophenyl ethers are nearly colorless and free *p*-nitrophenol is highly colored in alkaline solution, cleavage of the ethers is easily determined and in amounts as small as 0.02 micromoles.

Experimental. Under ethyl ether anesthesia the peritoneal cavity of a rat, 400-500 g in weight, was opened and exactly 0.5 millimoles of a *p*-nitrophenyl ether was deposited therein as a finely ground powder. In other experiments the compound was deposited subcutaneously. The weights of ethers used were: *p*-nitroanisole (the methyl ether) 76.5 mg; *p*-nitrophenetole (the ethyl ether) 83.5 mg; *p*-nitrodiphenyl ether, 130 mg. In 3 experiments 68.5 mg of *p*-nitrotoluene was used. The incision was sutured with silk. The urine was collected for 2 days using a cage and funnel device. The rats were not fed, but 15 cc of 0.9% sodium chloride was injected subcutaneously each day.

Both free and combined *p*-nitrophenol in the urine were determined. A standard curve was prepared relating varying amounts of recrystallized *p*-nitrophenol (from 0.05 to 3 micromoles in 10 cc of glycine buffer, pH 11.2) against the logarithm of the density of color. An Evelyn photoelectric colorimeter with 400 μ filter was used throughout.

Free *p*-nitrophenol was determined as follows: 1 cc of urine was acidified with 2N hydrochloric acid and extracted 3 times with about 10 cc of ethyl ether; the extract was transferred to a separatory funnel. The ether solution was twice washed with approximately 20 cc of water and then once with 10 cc of M/15 phosphate buffer pH 8. The ether solution was transferred to a colorimeter tube and 10 cc of glycine buffer pH 11.2 added. The tube was stoppered and shaken to deliver *p*-nitrophenol to the aqueous phase and depth of color determined colorimetrically.

The conjugated excretion products of *p*-nitrophenol were hydrolyzed before extraction. One ml of urine was added to 5 ml of Folin-Ciocalteu⁷ reagent and placed in a steam bath for 90 minutes and centrifuged; the total free

PNP was then determined as above on the supernatant.

Results. When 5 millimoles of the methyl and ethyl ethers of PNP were deposited intraperitoneally the rat suffered paralysis of the lower extremities with a profound fall of body temperature. However, the subcutaneous implantation of these and smaller amounts of the ethers was not followed by any evident toxic symptoms. Intraperitoneal injection of 0.5 mM amounts was not toxic.

A. Recovery of compound from urine. In normal rat urine, especially after acid hydrolysis, there appear to be traces of colored phenols and also small amounts of what are presumably carboxylic acids; these are extracted along with PNP by ether from acidic aqueous solution. Carboxylic acids, being more strongly acidic than PNP, are removed from the ether extract by shaking once with buffer of pH 8. This treatment removes only a trace or no PNP, although repeated extractions by a solution of pH 8 will remove a slight amount of PNP from the ether. Phenols which do not contain a nitro group are less acidic than PNP and remain in the ether phase when the PNP is finally extracted by buffer of pH 11. There was a 94-97% recovery of 0.25 and 0.5 micromoles of *p*-nitrophenol added to 1 ml samples of rat urine. The extraction of Folin-Ciocalteu reagent by the technic described yielded no color. The ethers of PNP were not broken down by steaming for 1½ hours in 2N hydrochloric acid or in Folin reagent. The time required for hydrolysis of conjugated PNP in urine by Folin-Ciocalteu reagent in the steam bath was determined at 15 minute intervals; maximum hydrolysis occurred after steaming for 1-1½ hours.

B. Cleavage by Intact Rat. The methyl and ethyl ethers of PNP were split at nearly the same rate (Table I) after intraperitoneal injection. The maximum excretion was between 6 and 24 hours after administration of the ethers and much greater amounts were split during the first than the second day. *p*-Nitrophenol was liberated mostly in the conjugated form; the ratio of conjugated to free PNP was in the order of 25 to 1. The methyl and ethyl ethers of PNP possess characteristic

⁷ Folin, O., and Ciocalteu, V., *J. Biol. Chem.*, 1927, **73**, 627.

TABLE I.
p-Nitrophenol in Urine After Phenyl Ether Administration.
 500 micromoles of the phenyl ether inserted intraperitoneally or subcutaneously.

Hours	0-6	%*	0-24	%*	24-48	%*	Total recovery	%*
			micromoles					
			(a) <i>p</i> -Nitroanisole.					
Free	0.9	0.18	1.59	0.32	0.32	0.06	1.91	0.38
Conj.	22.5	4.50	210.8	42.2	87.35	17.47	298.15	59.63
Free	5.69	1.14	13.47	2.70	0.85	0.17	14.32	2.86
Conj.	88.00	17.60	284.33	56.87	11.24	2.25	295.60	59.12
Free	1.54	0.31	3.20	0.64	0.70	0.14	3.90	0.78
Conj.	123.08	24.60	292.99	58.60	34.4	6.88	327.39	65.48
			(b) <i>p</i> -Nitrophenetole.					
Free	2.44	0.49	9.33	1.87	0.71	0.14	10.04	2.0
Conj.	86.10	17.22	220.3	44.06	4.80	6.96	255.10	51.02
Free	2.68	0.54	12.23	2.45	1.36	0.27	13.59	2.72
Conj.	83.41	16.68	206.15	41.23	52.10	10.42	258.25	51.65
Free	0.54	0.11	10.39	2.08	2.31	0.46	12.70	2.54
Conj.	55.38	11.08	204.29	40.86	69.03	13.81	273.22	54.64
			(c) <i>p</i> -Nitrodiphenyl ether.					
Free	0.16	0.03	0.64	0.13	0.34	0.07	0.98	0.2
Conj.	3.66	0.73	14.36	2.87	7.10	1.42	21.46	4.29
Free	0	—	0.27	0.05	0.60	0.12	0.87	0.18
Conj.	1.89	0.38	6.28	1.26	10.96	2.19	17.24	3.45

* % of ether excreted in urine as *p*-nitrophenol.

odors which were detectable in the urine after injection; while the unattacked ether seems from its odor to be excreted in the urine, no effort was made to determine it quantitatively in these experiments.

The use of *p*-nitrodiphenyl ether showed that the etherase effect was also operative on aromatic ethers. The splitting of the aromatic ethers was much less than ethyl and methyl ethers of *p*-nitrophenol (Table I) which might be expected from the fact that the carbon-oxygen bond in aromatic ethers is stronger than that of aliphatic ether. There was no detectable splitting of *p*-nitrotoluene.

C. Nature of excretory product. The combined ether extracts of the urine from 4 experiments with the methyl ether of PNP were pooled and evaporated to dryness. The compound was recrystallized twice from toluene yielding 109 mg of yellowish crystals, m.p. 112° (uncorrected). An intimate mixture of this substance with an authentic sample of *p*-nitrophenol (m.p. 112°) also melted at 112°. These results show that the excretory product obtained after administration of ethers

of *p*-nitrophenol is actually *p*-nitrophenol.

D. Enzymatic cleavage by tissue homogenates. In a preliminary study of the mechanisms of splitting of phenyl ethers by living tissues, homogenates of rat liver, kidney and pancreas 140 mg per 1 ml were prepared. Incubation at 37° for 22 hours was carried out of the following system: tissue homogenate 1 ml; M/15 phosphate buffer pH 7.4, 3 ml; methyl or ethyl ethers of PNP 2.5 micromoles dissolved in 5 ml of water containing 1% methyl alcohol. After 22 hours 20 ml of Folin-Ciocalteu reagent was added and the mixture digested for 90 minutes with extraction of free PNP as described above. Liver and kidney homogenates split 0.05 to 0.074 micromoles of free PNP (2-3% of the ether) which is enough to yield a definite yellow color. Pancreas was inactive. Preliminary boiling of the tissue homogenates abolished their capacity of cleavage; in control tubes without tissue or without PNP ethers no color was liberated.

Summary. Injection of methyl and ethyl ethers of *p*-nitrophenol in rats is followed by

excretion of large amounts of *p*-nitrophenol and its "conjugates" in the urine. This must involve a breakage of the carbon-oxygen bond of the ether linkage. Homogenates of rat kidney and liver but not of pancreas cause a definite though slight cleavage of these phenyl

ethers. Pancreas was inactive; boiling the liver and kidney extracts destroyed their activity against phenyl ethers. This evidence indicates that the breakdown of phenyl ethers by rat tissue is enzymatic and we propose for the enzyme the name phenyletherase.

16524

Nitrogen Retention after Intravenous and Oral Administration of Protein Hydrolysate and Native Protein Enterally.*

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Madden,¹ and Kozoll and Mok² have stated that of the two routes for supplying exogenous protein, the oral route is associated with greater nitrogen retention. Elman *et al.*³ and Shohl⁴ have indicated that a given protein digest is attended with the same nitrogen retention when supplied either parenterally or enterally. The addition of more experimental evidence on nitrogen retention under different modes of administration it was hoped might help resolve the conflicting published data.

Method. Mongrel dogs were used in this study. Dogs No. 4 and 5 were animals which had been placed on a low protein diet consisting essentially of apples, potatoes, carrots and sucrose. The diet was fortified with substantial amounts of vitamin B complex, cod liver oil, choline, and minerals. Caloric intake was limited to 40 calories per kilo body weight per day, and the studies were not begun until 30% of the original body weight had

been lost. Dog No. 6 was placed on the same diet and on the same caloric intake and the study started prior to any significant weight loss. Animals were kept in metabolism cages and urine collected over a 24-hour period, and specimens were analyzed quantitatively for total nitrogen by the macro Kjeldahl method.⁵ The amino acid nitrogen was determined by the method of Frame.⁶ Samples of the diet, and protein hydrolysate, and calcium caseinate (Casec[†]) were all analyzed for total nitrogen by the macro Kjeldahl method. At the beginning of each study period, the diet was colored with congo red and feces collected for total nitrogen determinations.

Protein hydrolysate (Amigen[†]) was given in amounts equivalent to 2 g of protein per kilo of body weight. This was administered in a total volume of 500 cc of fluid over a 6 hour period. After 5 days of intravenous infusion, the same amount of protein hydrolysate was given orally over a 6 hour period by means of a Wangenstein tube passed through the nose into the stomach. Two g of protein per kilo body weight as calcium caseinate was then given orally over a 6 hour period. A control period of 9 days was interposed between each

* The researches presented here were supported by grants of the Graduate School of the University of Minnesota.

† Fellow of the United States Public Health Service.

¹ Madden, S. C., Basset, S. H., Remington, J. H., Martin, F. J. D., Woods, R. R., and Skull, F. W., *Surg., Gyn., Obstet.*, 1946, **82**, 131.

² Kozoll, D., and Mok, W. T., *Proc. Central Soc. Clin. Research*, 1947, **20**, 12.

³ Elman, R., Archar, L. A., Horwitz, A., and Wolif, H., *Arch. Surg.*, 1942, **44**, 1064.

⁴ Shohl, A. T., *J. Clin. Invest.*, 1943, **22**, 257.

⁵ Peters, J., and Van Slyke, D. D., *Quant. Clin. Chem.*, 1931, **2**, 548.

⁶ Frame, E., *et al.*, *J. Biol. Chem.*, 1943, **199**, 225.

† Casec (calcium caseinate) and Amigen (enzymatic digest of casein) were kindly supplied by Mead Johnson of Evansville, Ind.

TABLE I.
Nitrogen Loss After Protein Hydrolysate Intravenously and Orally and After Calcium Caseinate Orally.
Dog. No. 4.*

No. of days	G. N. intake		G. N. output		Avg daily diff.	G. urine amino acid		Feces N.	
	Total	Avg daily	Total	Avg daily		Total	Avg daily	Total	Avg daily
9 control	4.30	0.48	5.09	0.57	-0.09	1.05	0.12	0.74	0.082
5 intrav. Amigent†	16.57	3.31	10.82	2.16	+1.15	2.14	0.43	1.15	0.23
9 control	4.30	0.48	7.81	0.87	-0.39	1.52	0.17	0.57	0.06
5 oral Amigen	16.57	3.31	9.21	1.84	+1.47	1.71	0.34	—	—
2 control	0.96	0.48	3.93	0.97	-1.49	0.60	0.30	—	—
5 oral Casec‡	16.57	3.31	6.83	1.37	+1.95	1.16	0.23	0.91	0.18

* Original wt, 13.55 kg; wt loss at start of study, 33.6%; wt loss at end of study, 44.0%.

† Casein hydrolysate equivalent to 2 g protein per kg body wt.

‡ Calcium caseinate equivalent to 2 g protein per kg body wt.

TABLE II.
Nitrogen Loss After Protein Hydrolysate Intravenously and Orally and After Calcium Caseinate Orally.
Dog No. 5.*

No. of days	Intake N in g		Urine N in g		Avg daily diff.	Urine amino acid N in g		Feces N	
	Total	Avg daily	Total	Avg daily		Total	Avg daily	Total	Avg daily
8 control	3.82	0.48	12.18	1.52	-1.04	1.21	0.15	0.73	0.09
6 Amigent† intrav.	21.49	3.58	10.99	1.83	+1.75	2.09	0.35	1.03	0.17
8 control	3.82	0.48	4.96	0.62	-0.14	0.90	0.11	0.72	0.09
5 Amigen orally	17.91	3.58	6.69	1.34	+2.24	1.61	0.32	0.74	0.17
9 control	4.30	0.48	5.55	0.62	-0.14	0.88	0.10	1.52	0.19
5 oral Casec‡	17.91	3.58	4.81	0.96	+2.62	0.48	0.10	0.26	0.05
5 control	2.39	0.48	2.92	0.58	-0.11	0.44	0.09	0.54	0.11

* Original wt, 14.7 kg; 34% wt loss at start of study; 45% wt loss at end of study.

† Casein hydrolysate equivalent to 2 g protein per kg body wt.

‡ Calcium caseinate equivalent to 2 g protein per kg body wt.

TABLE III.
Nitrogen Loss After Protein Hydrolysate Intravenously and Orally and After Calcium Caseinate Orally.
Dog No. 6.*

No. of days	Intake N in g		Urine N in g		Avg daily diff.	Urine amino acid N in g		Feces N	
	Total	Avg daily	Total	Avg daily		Total	Avg daily	Total	Avg daily
6 control	2.87	0.48	17.53	2.92	-2.34	6.30	1.05	—	—
5 Amigen intrav.	18.71	3.74	13.05	2.61	+1.13	2.41	0.48	1.31	0.26
9 control	4.30	0.48	9.14	1.02	-0.54	0.97	0.11	1.47	0.21
5 Amigen orally	18.71	3.74	10.08	2.02	+1.73	1.60	0.32	0.90	0.18
9 control	4.30	0.48	7.27	0.81	-0.33	1.78	0.20	0.30	0.03
5 oral Casec‡	18.71	3.74	8.21	1.64	+2.10	2.15	0.43	2.09	0.42
5 control	2.39	0.48	3.95	0.69	-0.21	0.52	0.10	0.86	0.17

* Wt, 10.2 kg; no wt loss at start of study; 21.6% wt loss at end of study.

† Casein hydrolysate equivalent to 2 g protein per kg body wt.

‡ Calcium caseinate equivalent to 2 g protein per kg body wt.

study period except in Dog No. 4 which had only 2 days of control between the oral protein hydrolysate and oral calcium caseinate. Urine

was collected during these periods and quantitative determinations made for nitrogen as during the test periods.

Results. The results are tabulated in terms of average daily nitrogen intake and average daily nitrogen excretion in the urine.

Dog No. 4, Table I, lost 2.16 g urinary nitrogen, or 65.4% of the intake after infusion of protein hydrolysate intravenously. 1.84 g urinary nitrogen or 55.6% of intake was lost when protein hydrolysate was given orally. 1.37 g urinary nitrogen, or 41.5% of intake was lost when calcium caseinate was given orally.

Dog No. 5, Table II, lost 1.83 g urinary nitrogen, or 51.2% of the intake after infusion of protein hydrolysate intravenously. 1.34 g of urinary nitrogen, or 37.3% of intake was lost when protein hydrolysate was given orally. 0.96 g urinary nitrogen, or 26.8% of the in-

take was lost when calcium caseinate was given orally.

Dog No. 6, Table III, lost 2.61 g urinary nitrogen, or 70% of intake when protein hydrolysate was given intravenously. 2.02 g urinary nitrogen, or 53.6% of intake was lost when protein hydrolysate was given orally. 1.64 g of urinary nitrogen, or 44% of the intake was lost when calcium caseinate was given orally.

Summary. It has been observed that with the oral ingestion of native protein (Casec), there is the greatest nitrogen retention; with the protein hydrolysate enterally there is less nitrogen retention; and with protein hydrolysate intravenously there is least nitrogen retention.

16525

Nitrogen Retention after Injection of Protein Hydrolysate into the Portal and Systemic Veins.*

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Numerous reports¹⁻³ suggest that positive nitrogen balance can be obtained by the parenteral administration of essential amino acid mixtures or protein hydrolysates. Regularly there is a sharp rise in the total urinary nitrogen following the infusion of these mixtures. Under these circumstances, theoretically, significant quantities of amino acids might be lost through renal excretory mechanisms before circulation through, and protein fabrication in, the liver could take place.

Method. Mongrel dogs were used in this study. Dogs No. 3, 4, and 5 were placed on a low protein diet consisting essentially of pota-

toes, apples, carrots, and sucrose. The diet was fortified with substantial quantities of vitamin B complex, cod liver oil, choline, and minerals. Caloric intake was limited to 40 calories per kg body weight daily. The animals were not studied until a weight loss of 30% had been reached. Dog No. 6 was placed on the low protein diet and the study begun prior to any significant weight loss. Caloric intake in this dog was similarly limited to 40 calories per kg of body weight.

Prior to the beginning of the study the animals were operated upon and a polythene tubing with an outside diameter of 1 millimeter and an inside diameter of 0.5 mm was threaded into a radicle of the splenic vein until it reached the entrance to the portal vein. The tube was brought to the outside through a separate stab wound. During infusion a blunt end 25 gauge needle was inserted into the tube. Four % sodium citrate solution was used to irrigate and fill the tube daily. A small glass plug was kept in the needle when not in use

* The researches presented here were supported by grants of the Graduate School of the University of Minnesota.

† Fellow of the United States Public Health Service.

¹ Elman, R., *Ann. Surg.*, 1940, **112**, 594.

² Brunswick, Al., Clark, D. E., and Corbin, N., *Ann. Surg.*, 1942, **115**, 1091.

³ Kremen, A. J., *Surgery*, 1948, **23**, 92.

TABLE I.
Nitrogen Loss After Administration of Protein Hydrolysate into the Portal and Systemic Veins.
Dog. No. 3.*

No. of days	N intake, g		N output, g		Avg daily diff.	Urine amino acid N, g	
	Total	Avg daily	Total	Avg daily		Total	Avg daily
7, control	3.35	0.48	4.15	0.59	-0.12	1.61	.23
5, Amigen† through portal vein	9.43	1.89	7.22	1.44	+0.44	1.72	.34
9, control	4.30	0.48	5.33	0.59	-0.11	0.88	.10
5, Amigen through peripheral vein	9.43	1.89	6.54	1.31	+0.58	1.02	.20

* Original wt, 13.55 kg; 33.6% wt loss at start of exp.; 44.0% wt loss at termination.

† Casein hydrolysate equivalent to 1 g protein per kg body wt.

TABLE II.
Nitrogen Loss After Administration of Protein Hydrolysate into the Portal and Systemic Veins.
Dog No. 4.*

No. of days	N intake, g		N output, g		Avg daily diff.	Urine amino acid N, g	
	Total	Avg daily	Total	Avg daily		Total	Avg daily
7, control	3.35	0.48	2.53	0.37	+0.11	—	—
5, Amigen† through portal vein	16.57	3.31	9.91	2.00	+1.33	1.78	0.36
9, control	4.30	0.48	5.09	0.57	-0.09	1.05	0.12
5, intravenous Amigen	16.57	3.31	10.82	2.16	+1.15	2.14	0.43
9, control	4.30	0.48	7.81	0.87	-0.39	1.52	0.17

* Original wt, 13.55 kg; wt loss at start of exp., 33.6%; wt loss at end of exp., 37.0%.

† Casein hydrolysate equivalent to 2 g protein per kg body wt.

and the abdomen was protected with a heavy canvas binder.‡

Beginning one week post-operatively, urine was collected daily as a control period. Specimens were analyzed quantitatively by the macro Kjeldahl⁴ method for total nitrogen. The amino acid nitrogen was determined by the method of Frame.⁵ Each batch of diet and protein hydrolysate was analyzed for total nitrogen by the macro Kjeldahl method. One g of protein as protein hydrolysate (Amigen⁶) per kg of body weight was infused into Dog No. 3 and 2 g of protein as protein hydrolysate per kg body weight was infused into Dogs No. 4, 5, and 6. About 220 cc (on the aver-

age) of 10% solution of protein hydrolysate was diluted with distilled water to make a final volume of 500 cc and this was given over a 6 hour period, care being taken to maintain a uniform rate of infusion. After 5 to 6 days of infusion through the portal vein, the dogs were allowed to rest 9 days during which time urine was collected and analyzed for total urea, creatinine, and amino acid nitrogen. Protein hydrolysate in the same volume and amount was then given intravenously through a vein in the foreleg over a 6 hour period. For the most part direct needling was used but when the animals were too restless, a polythene tubing was inserted into the vein. In no instance was there any untoward reaction noted. Dogs No. 5 and 6 had rectal temperatures taken after the hydrolysate was given through the portal vein and no febrile reaction was noted.

Results. The results are tabulated in terms of average daily nitrogen intake and average daily nitrogen excretion for each period.

Dog No. 3, Table I, lost 1.44 g of total urinary nitrogen or 76.7% of the total nitrogen

‡ Dog No. 4 autopsied at conclusion of the study showed no thrombus or phlebitis from prolonged use of an indwelling polythene tube. In Dogs 5 and 6 the tube was removed without subsequent bleeding and the dogs have remained healthy.

⁴ Peters, J., and Van Slyke, D. D., *Quant. Clin. Chem.*, 1931, **2**, 602.

⁵ Frame, E., et al., *J. Biol. Chem.*, 1943, **199**, 225.

⁶ Enzymatic casein hydrolysate kindly supplied by Mead Johnson and Co., Evansville, Ind.

TABLE III.
Nitrogen Loss After Administration of Protein Hydrolysate into the Portal and Systemic Veins.
Dog No. 5.*

No. of days	Intake N, g		Urine N, g		Avg daily diff.	Urine amino acid N, g	
	Total	Avg daily	Total	Avg daily		Total	Avg daily
7, control	3.35	0.48	6.89	0.98	-0.51	0.18	0.03
6, Amigent† through portal vein	21.49	3.58	15.77	2.63	+ .95	1.10	0.18
8, control	3.82	0.48	12.18	1.52	-1.04	1.21	0.15
6, Amigen intravenously	21.49	3.58	1.00	1.83	+1.75	2.08	0.35
8, control	3.82	0.48	4.96	0.62	-0.14	0.90	0.11

* Original wt, 14.7 kg; 34% wt loss at start of exp.; 35.4 wt loss at end of exp.

† Protein hydrolysate equivalent to 2 g protein per kg body wt.

TABLE IV.
Nitrogen Loss After Administration of Protein Hydrolysate into the Portal and Systemic Veins.
Dog No. 6.*

No. of days	Intake N, g		Urine N, g		Avg daily diff.	Urine amino acid N, g	
	Total	Avg daily	Total	Avg daily		Total	Avg daily
6, control	2.87	0.48	17.53	2.92	-2.44	0.63	0.11
6, Amigent† through portal vein	22.45	3.74	15.78	2.63	+1.11	2.79	0.46
8, control	3.82	0.48	8.06	1.01	-0.53	1.04	0.13
5, Amigen intravenously	18.71	3.74	13.05	2.61	+1.13	2.41	0.48
9, control	4.30	0.48	9.14	1.01	-0.54	0.98	0.11

* Weight, 10.2 kg; no wt loss at start of exp.; 14.5% wt loss at end of exp.

† Casein hydrolysate equivalent to 2 g protein per kg body wt.

intake during infusion of protein hydrolysate into the portal circuit. The urine amino acid nitrogen lost was 0.34 g or 18.4% of the total nitrogen intake. There was 1.30 g urinary nitrogen excretion or 69.3% of intake when protein hydrolysate was given into a peripheral vein. The urine amino acid nitrogen was 0.20 g or 10.9% of total nitrogen intake.

Dog No. 4, Table II, lost 1.98 g of total urinary nitrogen or 60% of the total nitrogen intake during infusion of protein hydrolysate into the portal circuit. The urine amino acid nitrogen lost was 0.36 g or 10.7% of the total nitrogen intake. There were 2.16 g of urine nitrogen or 65.5% intake when protein hydrolysate was given into a peripheral vein. The urine amino acid nitrogen lost was 0.43 g or 12.9% of the total nitrogen intake.

Dog No. 5, Table III, lost 2.63 g urinary total nitrogen or 73.7% of intake during infusion of protein hydrolysate into the portal circuit. The urine amino acid nitrogen lost was 0.18 g or 5.1% of total nitrogen intake.

There were 1.83 g of urine nitrogen or 51.4% of intake when protein hydrolysate was given into a peripheral vein. The urine amino acid nitrogen lost was 0.35 g or 9.7% of the total nitrogen intake.

Dog No. 6, Table IV, lost 2.63 g urinary nitrogen or 67.6% of intake during infusion of protein hydrolysate into the portal circuit. The urine amino acid nitrogen lost was 0.46 g or 12.4% of total nitrogen intake. There were 2.61 g urinary nitrogen or 69.7% of intake when protein hydrolysate was given into a peripheral vein. The urine amino acid nitrogen lost was 0.48 g or 12.9% of total nitrogen intake.

Conclusion. (1) No significant difference in the total nitrogen retention has been noted after infusion of protein hydrolysate into the portal or the systemic venous systems. (2) Polythene tube appears well tolerated and remains patulous for prolonged intravenous therapy in dogs.

16526

The Influence of Estrogens on Thyroid Function as Measured by Uptake of Radio-active Iodine.

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The influence of ovarian hormones on thyroid function has been the subject of many studies. It is generally believed that castration is followed by increased output of thyroid hormone, and that administration of estrogens depresses thyroid function. These changes are believed to be mediated by release and suppression of secretion, respectively, of thyrotrophic hormone as a result of the "shot gun" action of estrogens upon the anterior pituitary. Previous methods of investigation have included studies of the histology of the thyroid gland, determination of B.M.R., and determination of iodine in the blood.

Inasmuch as the uptake of iodine by the thyroid gland is a sensitive measure of its function, or, more precisely, of one specific phase of thyroid function, it appeared of interest to investigate the influence of estrogen administration and of castration upon the process.

Methods and materials. 159 female rats, descendants of Wistar strain, were used in this study. One group was injected with α -estradiol benzoate; a second group was spayed. In each experiment an equal number of animals served as controls.

Iodine uptake was determined by the use of a tracer dose of I^{131} . All rats, both experimental and control, were given an iodine-poor diet (Steenbock) and received 3.9 γ potassium iodide in 100 ml double distilled drinking water, following a procedure used previously by one of us.¹ This slightly iodine-deficient regime insures a more uniform and higher iodine uptake than that of rats on a stock diet, in which the uptake in controls may be too low to determine significant decreases in treated groups.

TABLE I.
Uptake of I^{131} by Thyroids of Ovariectomized Rats. Exp. 1.

	Spayed 50 days before	Controls
No. of rats	21	21
Days on diet*	14	14
Tracer dose of I^{131}	3.75 μ C	3.75 μ C
Uptake of I^{131} by thyroid in 4 hr: %	18.4 \pm 5.43%†	18.0 \pm 7.8%
Thyroid wt mg	11.5 \pm 1.76	11.3 \pm 2.37

* Steenbock diet with 3.9 γ % KI in drinking water.

† Standard deviation = $\sqrt{\frac{S(X)^2}{n-1}}$

At the end of the experiment all rats received I^{131} as sodium iodide intraperitoneally. Four hours after the injection the animals were killed and the thyroids removed and dropped into 3 ml of 2% sodium hydroxide solution. Radioactivity was determined on aliquots of the macerate of the thyroid glands by a Geiger counter.

Results. The findings are summarized in Tables I and II. In Experiment 1, the iodine uptake by the thyroid of rats spayed 64 days previously was compared with that of normal female controls, both groups being maintained on the experimental diet for 14 days prior to the time of sacrifice. There was no difference in either thyroid weight or uptake of I^{131} in these groups.

In Experiments 2, 3, and 4 (Table II) there was no significant difference in uptake of I^{131} between normal intact rats and those receiving estradiol benzoate. Fifty γ of the latter was administered 3 times weekly for 4 weeks (Experiment 2), 3½ weeks (Experiment 3), and one week (Experiment 4) to different groups.

Discussion. The extensive literature on the influence of ovarian hormones on thyroid

¹ Rawson, R. W., Tannheimer, J. F., and Peacock, W. C., *Endocrinology*, 1944, **34**, 245.

TABLE II.
Uptake of I¹³¹ by Thyroids of Rats Treated with Estradiol Benzoate.

	Exp. 2		Exp. 3		Exp. 4	
	T	C	T	C	T	C
No. of rats	23	17	17	17	22	21
Days on diet*	27	27	24	24	28	28
Estradiol benz.	12 × 50 γ = 600 γ (4 wks)	—	11 × 50 γ = 550 γ (3½ wks)	—	3 × 50 γ = 150 γ (1 wk)	—
Tracer dose of I ¹³¹	3.78 μ C	3.78 μ C	2 μ C	2 μ C	2 μ C	2 μ C
Uptake of I ¹³¹ by thyroid in 4 hr: %	33.7 σ† = 2.24	36.8 7.25	34.2 8.65	35.0 10.2	10.97 4.39	14.95 6.60
Thyroid wt mg	12.0 σ† = 2.24	13.1 2.75				

* Steenbock diet, 3.9 γ % KI in drinking water.

† Standard deviation = $\sqrt{\frac{S(X)^2}{n-1}}$

function and structure contains contradictory observations, reviewed by Salter² and Farbman.³ The only general agreement appears to be that ovarian secretions exert some influence on the thyroid.

The data presented here indicate that there is no difference in the uptake of a tracer dose of I¹³¹ in female rats 64 days after castration as compared with normal female controls. Moreover, injection of estradiol benzoate in quantities of 150 to 600 γ over 1 to 4 weeks failed to influence uptake of I¹³¹.

The uptake by the thyroid of a tracer dose of radioactive iodine is generally regarded as a measure of thyroid function; it has been shown that the uptake is increased in conditions of increased function and is decreased in conditions of hypofunction of the thyroid.

The following points should be borne in mind in interpreting these negative results and in comparing them with various reports in the literature.

The synthesis, storage, release and peripheral metabolic action of thyroid hormone constitute a complex chain of events. Uptake of iodide by the thyroid gland is the first link in this chain. Obviously, without adequate uptake of iodide there can be no adequate hormone synthesis, but equal uptake in two types

of experiments does not eliminate the possibility that differences may exist in the several further links of the chain. Thus it has been shown that iodine is taken up by the thyroid of thiouracil-treated rats but is not utilized for purposes of hormone synthesis. This evidently is not what is happening in the estrogen treated animals, but serves merely to indicate that study of one phase of thyroid functional activity does not permit final conclusions regarding other phases. Furthermore, there is good evidence that prolonged treatment with larger doses of estrogen, both natural and synthetic, do suppress the pituitary-thyroid "axis." The time factor may therefore play a significant role in this connection.

For reasons mentioned above the present studies were conducted under conditions of a slight iodine deficiency. This deficiency was not sufficiently severe to be goitrogenous as evidenced by the normal weight of the thyroids, but did make the glands more avid for iodine than those of rats kept on a stock diet. The uptake of tracer iodine was comparatively high, and the response to castration, or to the small amounts of estrogen employed in our experiments might be different at different levels of dietary iodine.

Summary. Uptake of tracer doses of I¹³¹ by rats' thyroids was not influenced by ovariectomy or by administration of estrogenic hormone.

² Salter, W. T., *The Endocrine Function of Iodine*, 1940.

³ Farbman, A. A., *J. Clin. Endocrin.*, 1944, **4**, 17.

16527 P

Some Experimental Evidence of Production of Adrenotrophin by the Fetal Hypophysis.*

L. J. WELLS (with the assistance of Dorothy N. Highby.)

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The relatively enormous size of the suprarenals in the human fetus raises the question as to whether the growth of these structures is normally governed in part by the action of adrenotrophin. Assuming that there might be more than one source of the adrenotrophin of pregnancy, it still remains to be shown whether the fetal hypophysis is a source. Accordingly, it was decided to approach the problem through experiments in rats.

Fifteen accurately timed fetuses with placental circulation intact were given a series of subcutaneous injections¹ of adrenotrophin[†] (Group A), 23 were deprived of the hypophysis by decapitation² (Group B) and 6 were subjected to both treatments (Group C). Near term they were taken by Caesarian section, weighed and autopsied. After each partially eviscerated body had been fixed in Bouin's solution, the lumbar region with the adrenals *in situ* was serially sectioned, and the sections stained either with hematoxylin and eosin or by the method of Dominici. Sections through adrenals of experimental fetuses were compared with those of controls by means of a microcomparator attached to two microscopes placed side by side. Thereafter, in each case, the volume of one adrenal was determined from the serial sections, using the paper-weight method, and the relative volume was calculated as follows:

$$\text{Rel. vol. adr.} = \frac{\frac{\text{Vol. adr. experimental fetus, mm}^3}{\text{Body wt experimental fetus, g}}}{\frac{\text{Vol. adr. littermate control, mm}^3}{\text{Body wt littermate control, g}}}$$

* Aided by grants from the American Philosophical Society, from Ciba Pharmaceutical Products, Inc., and from the Graduate Research Fund of the University of Minnesota.

¹ Wells, L. J., *Anat. Rec.*, 1946, **94**, 530.

The fetuses of Group A received a total of 0.05 to 0.8 mg of hormone, in 1 to 5 individual injections made about 12 hours apart, and were killed 14 to 54 hours after the initial injection. The relative volume of the adrenal was 1.08 to 3.36, the median being 2.34. Especially in those fetuses that received the larger doses, the cortical cells were exceedingly hypertrophic. The nuclei were significantly larger than in the controls, and the cytoplasm showed such increased granulation and vacuolation that it looked like fine foam. The outer zone of the adrenal, that of cellular proliferation and metamorphosis, showed many mitoses but was thin, giving the impression of having been rapidly "used up."

Fetuses of Group B, after having been deprived of the hypophysis, were either left inside the uterus or transferred to the maternal abdominal cavity, and were killed 47 to 102 hours later. In the 9 cases uncomplicated by any knowingly introduced variable,[‡] the relative volume of the adrenal, in terms of headless body weight of controls, was 0.39 to 0.80 (median, 0.60). The adrenal cortex was abnormally thin and failed to show the usual pattern of sinusoids and cellular cords, the cells being more tightly packed and much smaller than those of controls. Nuclei were abnormally small and the cytoplasm gave the impression of having been "washed out." The outer proliferative zone was relatively thick

[†] Generously supplied by the Armour Research Laboratories, Chicago (their Sample No. 32-D).

² Wells, L. J., *Anat. Rec.*, 1947, **97**, 409.

[‡] In the other 14 cases, all complicated by the fact that a littermate had received injections of adrenotrophin or of gonadotrophin (Upjohn's Gonadogen), the volumetric data were less consistent; thus in these cases it is necessary to consider the possibility that some injected hormone had actually crossed the placental barrier.

but quite condensed and its occasional mitotic figures were usually abortive.

The fetuses of Group C were operated and then given 0.25 to 0.5 mg adrenotrophin, in 4 to 5 divided doses, and were killed 49 to 54 hours after the first injection. Microscopically, the adrenal cortex resembled that of the fetuses of Group A. The relative volume of the adrenal, using operated littermates as controls, was 1.01 to 3.21, the theoretical median being 2.35. Also, the adrenals were much larger than those of normal littermates of the same sex (relative volume, up to 2.29).

In considering all aspects of the study, the

data show that injected hypophyseal adrenotrophin causes the fetal adrenal to grow more rapidly than in controls. They suggest that removal of the fetal head (hypophysis) inhibits growth of the adrenal and causes abnormal changes in the cortical cells. They indicate that this inhibition and these changes may be prevented by giving a series of injections of adrenotrophin.

In conclusion, these observations may be taken as experimental evidence of the production of adrenotrophin by the hypophysis of the fetal rat.

16528

Effect of Sulfamethazine on the Testes and Accessory Glands of Normal and Hypophysectomized Rats.

S. Y. P'AN. (Introduced by H. B. van Dyke.)

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Asplin and Boyland¹ have reported that chronic administration of sulfamethazine (4,6-dimethyl-2-sulfanilamidopyrimidine) causes a hyperplasia and increase in the weight of the testes and some increase of weight of the seminal vesicles and prostate in male rats receiving the drug from birth to a weight of about 50 g. In older males an increase in weight of only the seminal vesicles and the prostate was noted. They found that the drug had no effect on the seminal vesicles and prostate of castrated rats and postulated that sulfamethazine causes hyperplasia of the seminal vesicles and the prostate by stimulation of the testes.

Since the accessory glands of male rats are normally maintained and stimulated by androgen secreted by the interstitial tissue of the testes under the influence of the anterior pituitary gland, it seemed of interest to investigate whether the effects reported by Asplin and Boyland in young rats could be

secondary to the stimulating effect of the drug on the anterior pituitary gland. If that were the case, the ablation of the anterior pituitary gland would nullify the reported hypertrophy of the seminal vesicles and prostate in rats receiving sulfamethazine.

Experimental. Forty-one immature rats of weaning age (21-day old) of the same inbred stock were divided into 2 groups of 20 and 21 each. The hypophysis of the rats in the first group* was removed by the parapharyngeal approach according to the technic of Smith.² Half of the rats in both the hypophysectomized and the unoperated groups was placed on a stock laboratory diet. The other half of each group was fed on a similar diet to which 0.2% by weight of sulfamethazine† was added. The rats were weighed at the beginning of the ex-

* Four rats in this group died within one week after hypophysectomy.

² Smith, P. E., *Am. J. Anat.*, 1930, **45**, 205.

† Sulfamethazine was kindly furnished by Mr. W. A. Lott of the Squibb Institute for Medical Research.

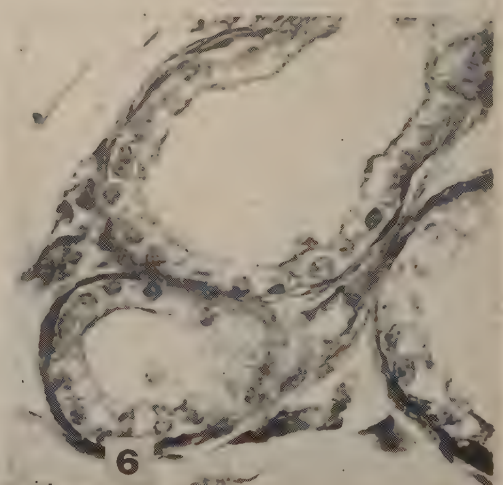
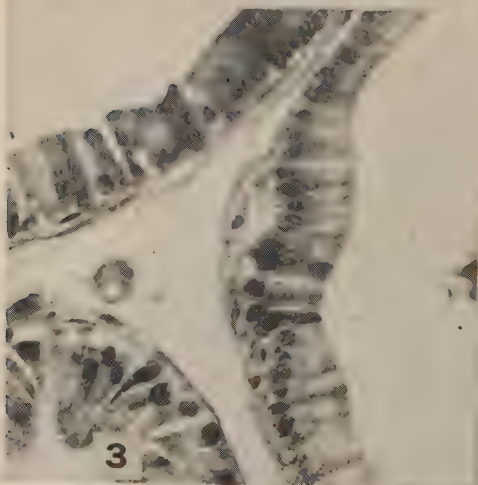
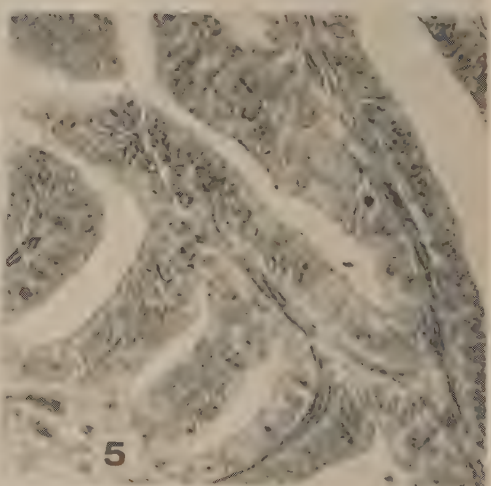
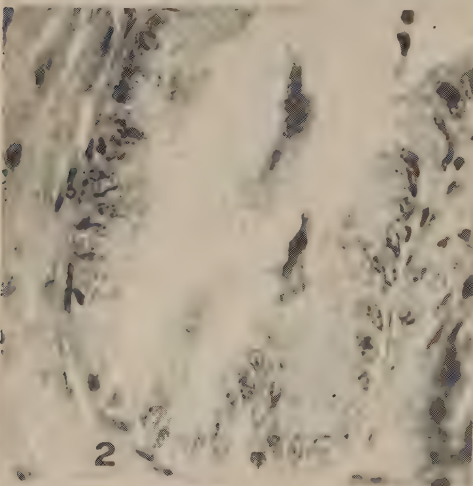
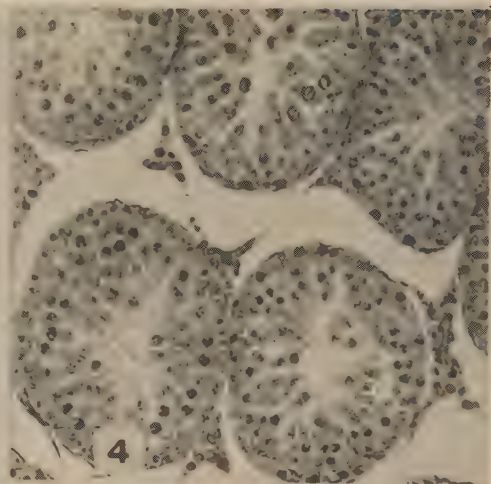
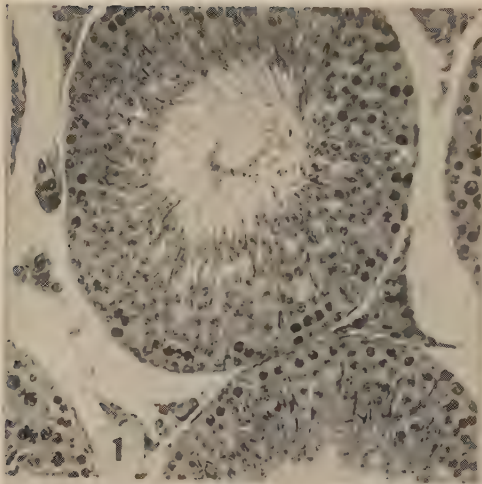
¹ Asplin, F. D., and Boyland, E., *Brit. J. Pharm. and Chemother.*, 1947, **2**, 79.

TABLE I.
The Effect of a Diet Containing Sulfamethazine on the Weight of Organs of Normal and Hypophysectomized Rats.

The Effect of a Diet Containing Sulfamethazine on the Weight of Organs of Normal and Hypophysectomized Rats									
			Body wt, g						
No. of rats	Type	Treatment	Start of exp.		End of exp.				
			Mean and S.D.	P	Mean and S.D.	P	Thyroid, mg	Adrenals, mg	P
8	Hypx		50.4 ± 2.9	>0.05	60.7 ± 5.7	>0.05	7.0 ± 1.6	8.6 ± 1.7	<0.01
8	"	Sulfameth.	47.5 ± 2.8		72.0 ± 10.8		5.6 ± 1.3	5.5 ± 1.2	
10	Normal		48.0 ± 8.6	>0.20	197.5 ± 25.4	<0.01	12.3 ± 3.4	14.6 ± 1.1	=0.3
11	"	"	44.5 ± 2.6		177.8 ± 18.1		10.8 ± 3.0	14.5 ± 1.5	

Wt of organs per 100 g body weight									
No. of rats	Type	Treatment	Testes, mg		P*	Seminal vesicles, mg		P	Anterior prostate, mg
			Mean and S.D.	P		Mean and S.D.	P		Mean and S.D.
8	Hypx		156.5 ± 45.1	<0.05	=0.2	7.0 ± 1.3	=0.4	7.3 ± 1.4	=0.05
8	"	Sulfameth.	117.3 ± 19.9			6.2 ± 1.2		6.8 ± 1.0	
10	Normal		1205.6 ± 97.1	=0.05	<0.01	105.2 ± 31.4	=0.02	67.5 ± 30.9	=0.3
11	"	"	1052.0 ± 205.5			50.2 ± 26.9		35.4 ± 24.2	

* P values (Fisher 1944) are those comparing the means of the groups in parentheses.



Rat No. 39700. Fed on stock laboratory diet, age 63 days at time of necropsy. Hematoxylin and eosin stains:

FIG. 1. Testes	× 200
FIG. 2. Seminal vesicle	× 400
FIG. 3. Anterior prostate	× 400

Rat No. 39829. Fed for 35 days on stock laboratory diet to which was added 0.2% of sulfamethazine, age 56 days at time of necropsy. Hematoxylin and eosin stains:

FIG. 4. Testes	× 200
FIG. 5. Seminal vesicle	× 400
FIG. 6. Anterior prostate	× 400

periment and twice weekly thereafter. To be sure of adequate absorption, the concentration of free sulfamethazine in the blood of all rats treated with the drug was determined at intervals according to the micromethod of Bratton and Marshall.³ All rats were killed with ether at the age of 56 to 63 days. The testes, seminal vesicles, anterior prostate, thyroid, adrenal and anterior pituitary glands of all the rats were carefully dissected, freed of connective tissue and weighed on a sensitive torsion balance. Bouin's fluid was used as fixative and paraffin sections were made and stained with hematoxylin and eosin.

Results. It will be seen from Table I that in normal rats, fed on diet containing sulfamethazine, the weight of the testes, seminal vesicles and anterior prostate per 100 g body weight were significantly smaller than those of rats fed on stock laboratory diet. The weights of the thyroid, adrenal and anterior pituitary glands (average 2.7 ± 0.5 mg for those of rats fed on stock laboratory diet and 2.8 ± 0.4 mg for those of rats fed on same diet to which 0.2% of sulfamethazine was added) of the normal rats fed on diet with or without sulfamethazine did not show any significant difference. A definite retardation of growth was noted in the normal rats dosed with sulfamethazine. In hypophysectomized rats, the testes, thyroid, and adrenals from animals receiving sulfamethazine were lighter than the same organs removed from control rats. The concentration of free sulfamethazine in the blood of both the normal and hypophysectomized rats receiving the drug varied from 5 to 13.5 mg per 100 cc of blood.

Microscopically, in the normal rats that were fed on diet containing sulfamethazine the most striking changes were seen in the testes

(Fig. 1 and 4). The tubules were much smaller in diameter than those of their littermate controls. There was complete absence of spermatogenic activities as evident by the total lack of spermatozoa. The interstitial tissue, however, did not seem to exhibit any pathological changes as compared with that in the testes of the untreated normal rats. The epithelial cells of the seminal vesicle in the treated rats showed a definite decrease in height. It will be seen in Fig. 2 and 5 that while the nuclei of the epithelial cells of the seminal vesicles of the untreated control rats occupy one-half or less of the cells, those of the epithelium of the sulfamethazine treated rats occupy two-thirds or more of the cells. Similar changes, although at places were not as marked as those seen in the seminal vesicle, were present in the epithelium of the anterior prostate gland. There were also found in the epithelium numerous vacuoles which are indicative of degenerative changes in the organ (Fig. 3 and 6). Although not accompanied by any significant change of weight, there were hyperplasia of the epithelium and vacuolation of the colloid in the acini of the thyroid of the normal rats receiving sulfamethazine in their diet. Many of the acini were obliterated. No significant change could be seen in the sections of the adrenal glands. In the hypophysectomized rats the testes of sulfamethazine-treated rats showed further degenerative changes, *i.e.* decrease in the number of germinal cells and increase of Sertoli cells in the germinal epithelium, compared with those of rats fed on stock laboratory diet. No important difference was noted in the sections of the seminal vesicles, anterior prostate, thyroid, or adrenal glands of the hypophysectomized rats fed on diet with or without sulfamethazine.

Discussion. It is evident from the foregoing data that contrary to the observations of

³ Bratton, C., and Marshall, E. K., *J. Biol. Chem.*, 1939, **128**, 537.

Asplin and Boyland,¹ the chronic administration of sulfamethazine to young male rats caused atrophy of the testes and accessory glands. The basis for the discrepancy is not clear and it is not believed that the higher concentration of sulfamethazine in the diet (0.2% instead of 0.1%) of the experiments here reported accounts for the difference in results. Since atrophic changes of the testes were observed both grossly and microscopically in the present experiments in both the normal and hypophysectomized rats chronically fed with sulfamethazine, it would appear that the effect of the drug on the gonads of the young rats is one of direct inhibition. Although the interstitial tissue of the testes of the sulfamethazine-treated normal rats did not exhibit any histopathological changes, the atrophic changes in the accessory glands in these rats may still be considered to be secondary to a decreased secretory activity, without any detectable histological changes in the interstitial tissue.

Except that there was no significant change in weight (per 100 g body weight), the hyperplasia of the thyroid gland of the sulfamethazine-treated normal rats conformed with the observations of MacKenzies⁴ and Ast-

wood⁵ who have reported that sulfonamides cause hyperplasia of thyroid glands in rats provided that the pituitary is intact. It remains to be determined, however, what significance should be attached to the apparent decrease in weights of the thyroid and adrenal glands in the hypophysectomized rats treated with sulfamethazine.

Summary. Chronic administration of sulfamethazine to normal male rats causes gross and microscopic atrophic changes in the testes, seminal vesicles and anterior prostate but a hyperplasia of the thyroid gland. The reported hypertrophy of the accessory organs¹ of the young male rats treated with sulfamethazine was not confirmed. When hypophysectomized rats were fed with sulfamethazine, only increased atrophy of the testes was manifested. The atrophic changes that occurred in the testes and accessory organs in normal rats, as well as the similar changes in the testes of the hypophysectomized male rats after chronic administration of sulfamethazine, are attributed to a direct effect of the drug on the testes.

The author wishes to express his indebtedness to Dr. H. B. van Dyke for suggestions and advice in carrying out the experiments, and Dr. P. E. Smith in examining the histopathological sections.

⁴ MacKenzie, C. G., and MacKenzie, J. B., *Endocrinology*, 1943, **32**, 185.

⁵ Astwood, E. B., Sullivan, J., Bissell, A., and Tyslowitz, R., *Endocrinology*, 1943, **32**, 210.

16529

Histochemical Variations in the Metrial Gland of the Rat During Pregnancy and Lactation.

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The metrial gland of the rat develops during pregnancy in association with the uterine blood vessels in the region of attachment of the mesometrium to the uterus. Some phases

of the histology of the metrial gland have been investigated.¹⁻⁴ Since little is known concerning the possible endocrine function of the

* The author wishes to express his appreciation to Miss Barbara Piper for her technical assistance.

¹ Goldmann, E. E., *Beitr. z. klin. Chir.*, 1912, **78**, 1.

² Gérard, P., *C. E. Soc. de Biol.*, 1925, **93**, 457.

³ Selye, H., and McKeown, T., *Proc. Roy. Soc., London, Series B*, 1935, **119**, 1.

⁴ Asplund, J., Borell, U., and Holmgren, H., *Z. f. mikr.-anat. Forsch.*, 1940, **48**, 478.

various parts of the rodent placenta, it was felt that a histochemical study of the metrial gland at various stages of pregnancy and lactation might give some indication of its function.

Methods. Virgin female rats were placed with males and their vaginal contents examined microscopically for spermatazoa not more than 18 hours later. Pregnancy was timed from the hour at which spermatazoa were found. Specimens were secured from over 61 rats from the fifth day of gestation to the twenty-second day of lactation. The metrial glands were fixed in Zenker-formol, picric-alcohol-formol, 10% neutral formalin, Carnoy's and Bouin's fluids. The following stains were employed: Masson, eosin and methylene blue and Best's carmine. Frozen sections of formalin-fixed tissues were studied for lipids by staining with Sudan red, Sudan black B, Nile blue sulfate and by the use of Schultz and plasmas reactions, polarizing microscopy and solubility tests. The Gomori⁵ procedure was employed for alkaline phosphatase and Perls' reaction for iron.

Results. The history of the metrial gland during pregnancy and lactation may be divided into 3 overlapping periods, each of these being marked by one or more outstanding histochemical characteristics. These periods were indicated, respectively, by the presence of (1) basophilia, (2) eosinophilic granules and glycogen, and (3) lipids.

Period of basophilia. As early as the sixth day of pregnancy, many of the mesenchyme-like connective tissue cells of the metrial gland area became basophilic and began to round up. This basophilia increased significantly during the next several days and was accompanied by cellular enlargement and hyperplasia (Fig. 2). The nucleolus hypertrophied and came into contact with the nuclear membrane. Digestion with crystalline ribonuclease[†] showed the basophilia of both cytoplasm and nucleolus to be due to the pres-

ence of ribonucleic acid.

This period was comparatively brief, the basophilia declining coincidentally with the development of eosinophilic granules and glycogen in these basophilic cells. At the height of the second period, basophilic material remained only as scattered clumps at the periphery of the granules and no young basophilic cells in the pre-granulated stage were to be found.

It should be noted that the first evidence of formation of the metrial gland (basophilia) appeared almost contemporaneously with the development of the decidual reaction in the uterine mucosa. In fact, basophilic cells with characteristics like those described above for the metrial gland also were found scattered among the typical decidual cells of the decidua basalis. The decidual basalis and the metrial gland were anatomically close to each other, being separated by the discontinuous inner circular layer of the tunica muscularis (Fig. 1). Thus, it appears that the same stimulus causes the formation of both the metrial gland and the decidua, such a conclusion being borne out by the artificial induction of deciduoma and metrial gland by Selye and McKeown.³

Period of eosinophilic granules and glycogen. The first appearance of eosinophilic granules in the basophilic cells occurred in the decidua basalis on the eighth day. On the ninth and tenth days, such granulating cells were found also on the metrial gland side of the circular layer of the tunica muscularis but were still more numerous in the decidua basalis. Henceforth, the decidua basalis atrophied and lost its basophilia. Meanwhile, eosinophilic granules developed rapidly in the metrial gland, these cells becoming arranged in cuffs around the blood vessels of the mesometrial attachment (Fig. 1 and 3). Soon after the appearance of the granules, glycogen formed in the periphery of the cell with the eosinophilic granules being grouped close to the nucleus. Both constituents reached their peak at the thirteenth to fifteenth day. During this period, the endothelium of the metrial gland blood vessels became columnar in shape and faintly basophilic (Fig. 3), this transformation generally not extending completely

⁵ Gomori, G., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 23.

[†] We wish to thank Dr. Moses Kunitz, the Rockefeller Institute for Medical Research, for supplying us with crystalline ribonuclease.

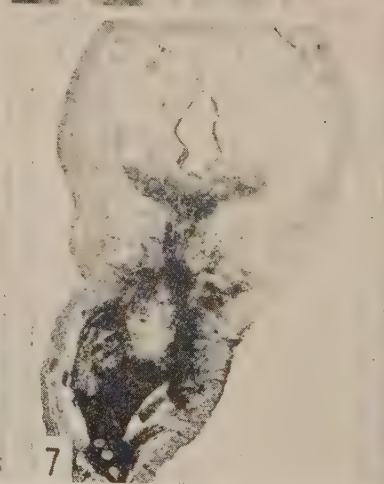
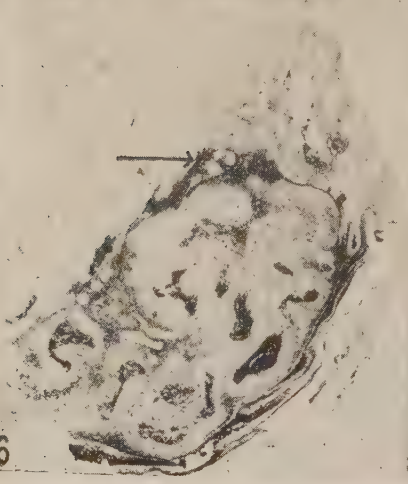
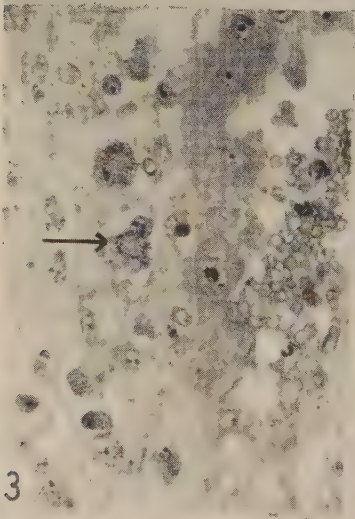
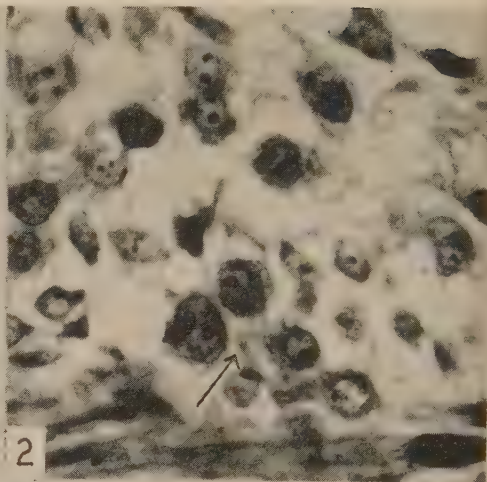
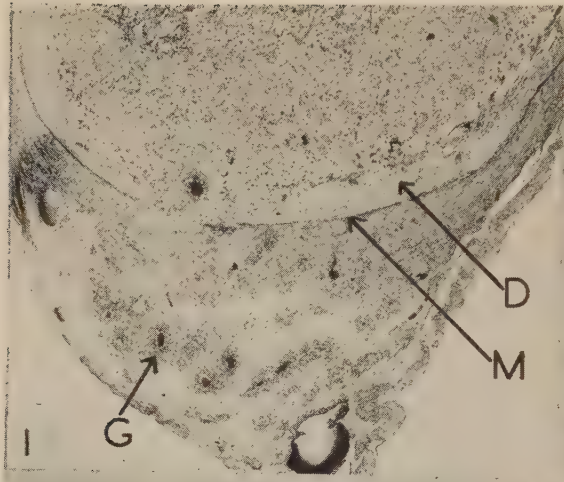


FIG. 1. Metrial gland (below M) at height of its development (fourteenth day of pregnancy). D—decidua basalis; M—inner layer of tunic muscularis; G—cuff of granulated cells around blood vessel.

FIG. 2. Basophilic cells (arrow). Eosin and methylene blue. Ninth day of pregnancy.

FIG. 3. Eosinophilic granules and glycogen vacuoles. Columnar endothelium. Thirteenth day of pregnancy.

FIG. 4. Alkaline phosphatase in columnar endothelium. Fourteenth day of pregnancy.

FIG. 5. Lipid at periphery of blood vessel. Nineteenth day of pregnancy. L—lumen.

FIG. 6. Lipid (arrow) at periphery of hyalinized vessel walls. Third day of lactation.

FIG. 7. Lipid (black) at fifth day of lactation. Sudan black B.

FIG. 8. Lipid at twenty-second day of lactation. Sudan red.

around the lumen. Intense alkaline phosphatase activity was demonstrated in such cells (Fig. 4). Subsequently, the eosinophilic granules and glycogen exhibited a steady decline and by the twenty-first day of pregnancy, both constituents were reduced significantly in amount.

The granulated cells met a varied fate. Some underwent necrosis *in situ*. Others were found in the lumina of the metrial gland blood vessels, apparently being carried toward the fetus.

Period of lipids. Lipid appeared on the seventeenth day of pregnancy at the periphery of the cuffs of granulated cells surrounding the metrial gland blood vessels (Fig. 5). Thus, it arose contemporaneously with the decline in glycogen and eosinophilic granules. The lipid increased slightly in quantity during the remainder of pregnancy and early lactation (Fig. 6) and remained located chiefly in the connective tissue between blood vessels. However, by the fifth day of lactation, the lipid-containing cells had formed a compact body (Fig. 7) containing an intricate network of capillaries. These cells were multicellular with vacuoles of varied size and possessed a central or slightly eccentric nucleus. Pigment appeared for which a hematogenous origin was indicated by a positive reaction for iron and failure to stain sudanophilically. In other cases, some of the pigment did appear to stain with Sudan black, indicating it to be lipochrome. The metrial gland remained without great change until the twenty-second day of lactation (Fig. 8).

The lipid gave a positive Schultz reaction, stained rose with Nile blue sulfate and re-colorized the Schiff reagent without pretreatment with mercuric chloride. At least a part of it was birefringent and some of it

showed the black cross of polarization. Much of it was acetone-soluble. This information suggests that the lipid was a mixture containing at least glycerides (some of which were unsaturated) and cholesterol or its esters.

Discussion. Ribonucleic acid was shown to be responsible for the early basophilia of the metrial gland. Cytoplasmic ribonucleic acid may be formed in the nucleolus⁶ and be involved in protein synthesis.⁷ Thus, the early prominence of basophilia in the metrial gland is to be correlated with rapid growth and hyperplasia of the organ and possibly also with the synthesis of whatever secretion may be represented by the eosinophilic granules of the second period.

Presumably the glycogen of the second period serves as nutriment for the embryo.^{2,3} Alkaline phosphatase in the columnar endothelium probably assists in glycogenesis by splitting hexosephosphate.

The lipid may be of varied significance. First, some of its histochemical reactions resemble those which characterize the steroid-secreting endocrine glands. Possibly the metrial gland contributes to the steroid metabolism of the rat at the time of parturition and during lactation. Second, the lipid may have resulted from involution of the metrial gland. Third, in the structure of the lipid-containing cells, the positive reaction for cholesterol and its esters⁸ and in its association with pigment, some of which may be lipochrome in type, the lipid of the metrial gland resembles brown fat. The physiology of

⁶ Caspersson, T., and Schultz, J., *Proc. Nat. Acad. Science*, 1940, **20**, 507.

⁷ Greenstein, J. P., *Advances in Protein Chemistry*, Academic Press, Inc., New York, 1944.

⁸ Cramer, W., *Brit. J. Exp. Path.*, 1920, **1**, 184.

brown fat is poorly understood but an endocrine function has been suggested for it.⁹

Summary. During pregnancy and lactation the metrial gland is marked successively by

⁹ Long, J. A., *Anat. Rec.*, 1922, **23**, 107.

the prominence of (1) ribonucleic acid, (2) eosinophilic granules and glycogen, and (3) lipids. These appear to be related, respectively, to growth and secretion, nutrition for the embryo and steroid metabolism.

16530

Effect of Bacterial Endotoxins on Glycogen Synthesis.*

ERNEST KUN. (Introduced by C. Phillip Miller.)

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It was found previously that the liver and muscle of rabbits which were poisoned with bacterial endotoxins showed a considerable depletion in glycogen.¹ Since the mechanism of this decrease in tissue glycogen was not known, further experiments were performed to investigate this problem. The experiments described in this paper deal with the effect of endotoxins on the synthetic process of glycogen formation from glucose and pyruvate *in vivo* and *in vitro*.

Experimental. The effect of Salmonella endotoxin on glycogen synthesis *in vivo* was studied in rats under the following experimental conditions:² Eighteen white rats, ranging in weight from 250 to 310 g, were starved for 48 hours. During this period the animals had free access to water. At the time of the experiment the animals were divided in 2 equal groups, one of which served as controls while the other was injected intraperitoneally with 2 ml (20 mg solid content) of Salmonella endotoxin per rat. It was found in preliminary experiments that this amount of endotoxin was lethal in 4-6 hours. One hour after injection of the endotoxin, 3 rats in each group were sacrificed and the liver glycogen determined.³ At the same time, the remaining 12 rats were given 3 ml of saturated glucose

TABLE I.
Effect of Salmonella Endotoxin on Glycogen Synthesis in the Livers of Rats.

Time	Liver glycogen (mg/g)	
	Normal	Poisoned*
Before glucose	1.74	0.18
	2.84	1.25
	2.21	0.97
2 hr after	16.2	0.39
	11.6	0.25
	15.8	0.36
3 " "	18.6	0.43
	16.9	0.44
	17.0	0.35

Each value obtained on one animal.

* Poisoned rats were injected with 2 ml (20 mg dry wt) of endotoxin one hour before glucose.

solution by stomach tube. Two and 3 hours after the administration of glucose 3 animals in each group were sacrificed and their livers analyzed for glycogen. As indicated in Table I, the liver glycogen of the normal rats increased 6-8 fold in 2-3 hours, while the rats injected with endotoxin showed no appreciable increase. It is thought that this effect of the endotoxin is not a non-specific protein effect, since the injection of 20 mg bovine plasma protein per rat had no measurable effect on liver glycogen synthesis.

The effect of endotoxins on glycogen synthesis *in vitro* was studied in rabbit liver slices

* This investigation was undertaken and supported jointly by the U. S. Navy, Office of Naval Research, and the University of Chicago.

¹ Kun, E., and Miller, C. P., *Proc. Soc. Exp. Biol. and Med.*, 1948, **67**, 221.

² Barron, E. S. G., personal communication.

³ Unbreit, W. W., Burris, R. H., and Stauffer, J. F., *Manometric Techniques and Related Methods for the Study of Tissue Metabolism* (Burgess Publishing Company), 1946, p. 170.

and in rat diaphragm. Rabbits were starved for 24 hours, then sacrificed and the liver slices kept in ice cold, substrate-free medium for 30 minutes. Five ml of the K-Ca medium of Buchanan, Hastings and Nesbett⁴ were measured into glass-stoppered centrifuge tubes containing 50 mg glucose or 10 mg lithium pyruvate as substrate and various amounts of endotoxins. Liver slices, weighing 250-350 mg each were placed in tubes containing substrate plus 5 ml of medium and aerated with 95% O₂ + 5% CO₂ for 3 minutes. The tubes were then closed with glass stoppers and shaken for 1 hour at 37°C. The reaction was then stopped by the addition of 5 ml 60% KOH and the tubes were heated in a boiling water bath for 15 minutes. The glycogen was determined as glucose.⁸ In each experiment liver slices were shaken in substrate-free medium simultaneously with the experimental tubes. The differences in glycogen content of these blanks and the experimental (containing substrate) were taken as a measure of glycogen synthesis. As shown in Tables II and III, there was less glycogen formed in the presence of meningococcal endotoxin than in the controls.

Since Gemill⁵ and Hamman,^{6,7} later Stadie

TABLE II.

Inhibition by Meningococcal Endotoxin of Glycogen Synthesis* from Glucose in Rabbit Liver Slices *in Vitro*.

No. of exp.	Normal, mg/g	Plus meningococcal endotoxin 0.1 mg/ml medium,	% inhibition
		mg/g	
1	2.6	1.36	55
2	3.1	0.88	72
3	2.1	1.11	47
4	1.9	0.36	81
5	2.7	1.2	56
6	2.4	0.96	60

* Glycogen synthesis is expressed in terms of mg glycogen formed in one hour per g of liver in 5 ml K-Ca medium in presence of 50 mg glucose substrate.

Gas—O₂. Temperature—37°C.

⁴ Buchanan, J. M., Hastings, A. B., and Nesbett, F. B., *J. Biol. Chem.*, 1942, **145**, 715.

⁵ Gemill, C. L., *The Johns Hopkins Hosp. Bull.*, 1940, **66**, 232.

⁶ Gemill, C. L., and Hamman, L., Jr., *The Johns Hopkins Hosp. Bull.*, 1941, **68**, 50.

TABLE III.

Inhibition by Meningococcal Endotoxin of Glycogen Synthesis* from Pyruvate in Rabbit Liver Slices.

No. of exp.	Liver, γ/g	Liver plus endotoxin, γ/g	% inhibition
1	805	530 (5 mg toxin)	32
2	1100	710 (5 " ")	35
3	990	480 (10 " ")	50
4	780	370 (10 " ")	52
5	970	510 (10 " ")	47

* Glycogen synthesis is expressed in terms of micrograms of glycogen per g of liver synthesized in one hour in presence of 10 mg Li-pyruvate in 5 ml K-Ca medium.

Gas—O₂. Temperature—37°C.

and Zapp,⁸ and Krahl and Cori⁹ had demonstrated that insulin increased the glucose uptake of the rat diaphragm *in vitro*, it was of interest to determine whether the action of insulin is affected by endotoxin and vice versa. In these experiments, the method described by Krahl and Cori⁹ was employed. This technic allows the use of pieces of the same diaphragm for experimental and control observations. Each experiment shown in Table IV gives the results obtained from strips of the same rat diaphragm. Insulin increased the glucose uptake of the rat diaphragm while the decrease of glucose utilization in the presence of *Salmonella* endotoxin was less in the presence of insulin.

Discussion. In all experiments, endotoxins of meningococcus and *Salmonella aertrycke* caused an inhibition of glycogen formation from pyruvate and glucose. The most striking effect could be observed *in vivo*, where this synthetic process was completely inhibited. These observations make it probable that the depletion of liver and muscle glycogen in toxic animals may be at least partially explained on this basis.

It is significant that Cross and Holmes¹⁰ observed similar inhibition of glycogen synthesis by diphtheria toxin. However, this

⁷ Gemill, C. L., and Hamman, L., Jr., *The Johns Hopkins Hosp. Bull.*, 1941, **68**, 329.

⁸ Stadie, W. C., and Zapp, J., Jr., *J. Biol. Chem.*, 1947, **170**, 55.

⁹ Krahl, M. E., and Cori, F. C., *J. Biol. Chem.*, 1947, **170**, 607.

¹⁰ Cross, M. C. A., and Holmes, E. G., *Brit. J. Exp. Path.*, 1937, **18**, 370.

TABLE IV.

Effect of Salmonella Endotoxin and Insulin on the Glucose Uptake of the Rat Diaphragm.

No. of exp.	Normal, mg/g	+Insulin 1 unit/ml, mg/g	+Toxin 0.1 mg/ml, mg/g	+Toxin-insulin, mg/g
1	3.44	4.4	2.1	4.0
2	2.91	3.92	1.9	3.1
3	4.89	5.1	3.1	4.6
4	3.94	4.6	0.1	3.7
5	4.95	5.4	3.2	4.4
6	2.81	4.21	2.0	2.6
7	2.5	3.40	0.6	1.6
8	4.7	6.1	2.2	4.8
9	3.9	5.1	1.9	2.8

Glucose uptake is expressed in terms of mg glucose per g of tissue utilized in 2 hours.

similarity does not mean that the mode of action of diphtheria toxin is necessarily the same as that of the endotoxins. It was recently found that the phosphorylation of glu-

cose was inhibited by meningococcal endotoxin.¹¹ This inhibition, together with the inhibition of the succinoxidase¹ might serve as an explanation of the inhibition of glycogen synthesis.

Summary. Intraperitoneal injection of Salmonella endotoxin completely inhibited *in vivo* glycogen synthesis from glucose in the liver of rats. In liver slices of rabbits, there was less glycogen formed *in vitro* from glucose and pyruvate in the presence of meningococcal endotoxin. The glucose uptake of rat diaphragm *in vitro* was decreased by Salmonella endotoxin and this inhibition of glucose utilization could be diminished by the addition of insulin.

¹¹ Kun, E., *J. Biol. Chem.*, 1948, **174**, 761.

16531

Influence of Crude Trypsin Inhibitor on Utilization of Hydrolyzed Protein.

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As a result of the reports of Ham and Sandstedt^{1,2} on the presence of a trypsin inhibitor in raw soybeans, there has been considerable interest regarding the possible role that this trypsin inhibitor plays in altering the nutritive value of proteins. A relationship between the well known phenomenon of increased nutritive value of soybean protein resulting from heat treatment and the destruction of trypsin inhibitor has been suggested by several investigators. In fact, an excellent correlation between nutritive value and trypsin inhibitor content was observed in soybean preparations heated under varying conditions by Westfall and Hauge.³ Melnick, Oser, and

Weiss⁴ observed that the relative rates of liberation of methionine, lysine and leucine as a result of tryptic digestion were different in raw soybeans and in soybeans in which the trypsin inhibitor had been inactivated by heat. They concluded from this study that the influence of the trypsin inhibitor on nutritive value of the soybean protein was due to an alteration in the time factors of availability of essential amino acids, particularly methionine, in the intestinal tract. Desikachar and De⁵ have provided some evidence that does not support the conclusions of Melnick and his associates. They compared the digestibility and biological value of unmodified raw soybeans with papain-digested soybeans to which a water extract of soybeans containing

¹ Ham, W., and Sandstedt, R. M., *J. Biol. Chem.*, 1944, **154**, 505.

² Ham, W., Sandstedt, R. M., and Mussehl, F. E., *J. Biol. Chem.*, 1945, **161**, 635.

³ Westfall, R. J., and Hauge, S. M., *J. Nutrition*, 1948, **35**, 379.

⁴ Melnick, D., Oser, B. L., and Weiss, S., *Science*, 1946, **103**, 326.

⁵ Desikachar, H. S. R., and De, S. S., *Science*, 1947, **106**, 422.

the trypsin inhibitor had been added so that the trypsin inhibitor content of both the digested and undigested samples was approximately the same. Both preparations showed the same digestibility and biological value and Desikachar and De concluded that there must be factors separate from the proteolytic inhibitor that affect the nutritive value of the soybean protein. In the work of these investigators the nutritive value of the papain digested soybean before supplementation with trypsin inhibitor was not measured. Therefore, the possibility remains that the addition of trypsin inhibitor to the digested protein did not alter the biological value of the protein. Since this important control was not included in this study it was considered advisable to reinvestigate the effect that soybean trypsin inhibitor has on enzymatically digested protein.

Methods. The hydrolyzed protein chosen for this study was Protolysate.* This hydrolysate represents the pancreatic enzyme digest of casein and other animal proteins. The extent of digestion is not complete since the free amino nitrogen represents approximately 65% of the total nitrogen. However, it is basically a tryptic digest in which hydrolysis has been carried farther by the action of naturally occurring exopeptidases. The trypsin inhibitor preparation was made according to the general directions described by Klose, Hill, and Fevold,⁶ and essentially is an ammonium sulfate precipitate of an acid extract of raw soybeans. The trypsin inhibitor activity of this extract was determined by a modification of the method of Charney and Tomarelli⁷ and the extract was added to either casein or Protolysate in such amounts that the trypsin inhibitor potency was approximately the same as that of raw soybeans. As an additional control the trypsin inhibitor extract was inactivated by autoclaving at 15 pounds pressure for 15 minutes and was added in the same

TABLE I.
Growth Response and Protein Efficiency Ratio (Grams Gain in Weight per Gram Protein Ingested) of Mice Receiving an Extract of Raw Soybeans Containing Trypsin Inhibitor.

Protein source	% prot. in diet (N × 6.25)	Avg initial wt, g	Avg final wt, g	Avg wt gain, g	Avg food consumed g/10 days	Avg protein consumed g/10 days	Protein efficiency	Relative protein efficiency
Casein	10.29	7.50	12.86	5.36	21.73	2.24	2.39	100
Casein + 1% inhibitor	11.54	7.57	11.46	3.89	21.10	2.43	1.60	67
Protolysate	10.84	7.30	12.01	4.71	20.83	2.26	2.08	87
Protolysate + inhibitor (1%)	11.30	7.26	9.81	2.56	21.51	2.43	1.05	44
Protolysate + inhibitor (1%) autoclaved	11.29	7.47	12.96	5.49	24.90	2.81	1.95	82

* Trademark of a protein hydrolysate preparation purchased from Mead, Johnson and Company.

⁶ Klose, A. A., Hill, B., and Fevold, H. L., *Proc. Soc. Exp. Biol. and Med.*, 1946, **62**, 10.

⁷ Charney, J., and Tomarelli, R. M., *J. Biol. Chem.*, 1947, **171**, 501.

amount to the Protolysate.

Diets containing casein or Protolysate as the source of protein were fed *ad libitum* to groups of 7 male albino mice. General details of this type of feeding experiment including diet composition have been described by Bosshardt *et al.*⁸ Growth rate and food consumption were measured for 10 days. The addition of the active or autoclaved extract as 1% of the diet increased the protein content of the diets as is shown in Table I.

Results. The results in Table I show that addition of the trypsin inhibitor extract to casein markedly reduced growth and the protein efficiency ratio. This confirms the observations of various investigators.^{2,6} The addition of the active extract to the hydrolyzed protein also resulted in a decrease in growth rate and protein efficiency ratio. However, the autoclaved extract was without appreciable effect. It is interesting to note that the harmful effects of the active soybean extract were not associated with a decrease in food consumption. This is contrary to many observations with raw soybeans.

These results confirm and extend the observations of Desikachar and De. They do not support the conclusion of Melnick *et al.* that the activity of the trypsin inhibitor in lowering the nutritive value of proteins is due to an alteration in the intestinal digestion of protein. The general conclusion can be drawn

that an extract of raw soybeans containing active trypsin inhibitor is deleterious to the growth rate and protein utilization of mice receiving as a sole source of protein in the diet a well hydrolyzed protein. Since the harmful effect of the extract was destroyed by autoclaving, it can be concluded further that the deleterious factor, whether it be trypsin inhibitor or some other inhibitory substance of soybeans, is heat labile. The fact that the soybean extract containing trypsin inhibitor decreased the utilization of a hydrolyzed protein cannot in itself be used as evidence that the trypsin inhibitor is not the factor responsible for the poor growth response and utilization of intact protein. There are many possibilities of indirect mechanisms whereby an inhibitor of proteolytic activity could exert a deleterious effect in the intestinal tract or in the animal body aside from the inhibition of tryptic digestion of dietary protein within the intestinal tract.

Summary. The addition of an extract of raw soybeans containing active trypsin inhibitor to an enzymatic protein digest (Protolysate) caused a decrease in growth rate and protein utilization. Autoclaving, which inactivated the trypsin inhibitor, abolished the harmful effects of the extract. It has been concluded that the deleterious effects resulting from the ingestion of an extract of raw soybeans are not necessarily due to an interference with the intestinal digestion of dietary protein.

⁸ Bosshardt, D. K., Ydse, L. C., Ayres, M. M., and Barnes, R. H., *J. Nutrition*, 1946, **31**, 23.

Toxicity of 1-(3,4-Dihydroxyphenyl)-2-Isopropylaminoethanol Hydrochloride (Isuprel).

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Konzett,¹ Lands, Nash, McCarthy, Granger and Dertinger,² and Siegmund, Granger and Lands³ have described the pharmacologic actions of 1-(3, 4-dihydroxyphenyl)-2-isopropylaminoethanol HCl (Isuprel*). Stolzenberger-Seidel,⁴ Dautrebande *et al.*,⁵ and Segal and Beakey⁶ have shown this substance to be an effective bronchodilator in bronchial asthma. This communication reports a series of toxicity studies carried out in this laboratory in an effort to evaluate better this new therapeutic agent.

Acute Toxicity. Albino mice, weighing 15-21 g, raised in our laboratory, were used for the determination of acute toxicity. The test animals were housed in the colony room for the duration of the experiment at a constant temperature of 76°F. Isuprel was injected intraperitoneally and the resultant deaths taking place during the 72 hours following injection were recorded. Epinephrine, arterenol, and racemic epinephrine, similarly administered, were injected into a comparable number of mice in order to compare the relative toxicity of these substances with that of Isuprel. The data were calculated by the method of Miller and Tainter,⁷ with the results

shown in Table I.

Isuprel was found to have an LD₅₀ of 494 mg/kg when injected intraperitoneally, as compared with 4.6 mg/kg for epinephrine, 15.6 mg/kg for arterenol, and 7.8 mg/kg for racemic epinephrine. Arterenol appears to be 38 times and epinephrine 107 times more toxic than Isuprel. Doses as large as 720 mg/kg of Isuprel did not cause death in some instances. Acute 24 hour intravenous toxicities were determined in albino mice. These data also show the relatively low toxicity of Isuprel. Racemic epinephrine was found to be 16 times and arterenol 7.8 times more toxic than Isuprel.

Albino rats taken from our own colony and housed under the same environmental conditions as the colony animals were given Isuprel by subcutaneous injection or orally by intubation. Results obtained are included in Table I. Rats were injected subcutaneously with a dose as large as 100 mg/kg without the appearance of toxic effects. Richards⁸ has reported the LD₅₀ of epinephrine to be 5.3 mg/kg when administered subcutaneously in rats, while West⁹ has reported a value of 12 mg/kg for the same compound. Arterenol, with an LD₅₀ of less than 2.0 mg/kg, is even more toxic when administered in this manner (Schulte *et al.*).¹⁰ Isuprel, administered by subcutaneous injection, was found much less toxic than these structurally similar compounds.

When given by intubation to rats, Isuprel

¹ Konzett, H., *Arch. f. exp. Path. u. Pharmacol.*, 1940, **197**, 27.

² Lands, A. M., Nash, V. L., McCarthy, H. M., Granger, H. R., and Dertinger, B. L., *J. Pharm. and Exp. Therap.*, 1947, **90**, 110.

³ Siegmund, O. H., Granger, H. R., and Lands, A. M., *J. Pharm. and Exp. Therap.*, 1947, **90**, 254.

* Isuprel is the registered trade mark of Winthrop-Stearns, Inc.

⁴ Stoltzenberger-Seidel, M., *Klin. Wochenschr.*, 1940, **51**, 1306.

⁵ Dautrebande, L., Philippot, E., Charlier, R., Dumoulin, E., and Nogarede, F., *Arch. internat. Pharmacodyn. Therap.*, 1942, **68**, 117.

⁶ Segal, M. S., and Beakey, J. F., *Bull. New Eng. Med. Cen.*, 1947, **IX**, 62.

⁷ Miller, L. C., and Tainter, M. L., *Proc. Soc. Exp. Biol. and Med.*, 1944, **57**, 261.

⁸ Richards, R. K., *J. Pharm. and Exp. Therap.*, 1943, **79**, 111.

⁹ West, G. B., *Nature*, 1945, **155**, 20.

¹⁰ Schulte, J. W., Reif, E. C., Bacher, J. A., Lawrence, W. S., and Tainter, M. L., *J. Pharm. and Exp. Therap.*, 1941, **71**, 62.

TABLE I.
 Acute Toxicity of Isuprel and Related Compounds.

Drug	Albino species	Route	No. animals	LD ₁₀	Toxicity mg/kg LD ₅₀ ± S.E.	LD ₉₀
Racemic Isuprel	Mice	Intrap.	273	377	494 ± 13.9	650
		Intrav.*	30	57.7	77 ± 7.0	99
	Rats	Subcut.	22	>100	>2000	
		Oral	20			
Epinephrine	Mice	Intrap.	80	2.15	4.6 ± 0.55	9.9
		Intrav.*	30	1.99	3.14 ± 0.23	4.8
	Rats	Subcut. ⁸	96		5.3	
		Subcut. ⁹			12	
Racemic Arterenol	Mice	Intrap.	72	4.6	15.6 ± 3.8	53.5
		Intrav.*	80	3.0	9.8 ± 1.9	27.4
	Rats	Subcut. ¹⁰	10	<2.0	>2.0	
Racemic Epinephrine	Mice	Intrap.	80	2.92	7.8 ± 1.3	21
		Intrav.*	30	2.5	4.8 ± 1.08	9.3
	Rats	Subcut.	14	>15	<20	

* Values for 24-hour LD₅₀.

was found to be tolerated by some animals in doses as large as 2000 mg/kg. Toxic symptoms observed were salivation and nervousness, followed by depression. Four of 10 animals given 1800 mg/kg died within 24 hours after drug administration.

Acute oral and intramuscular toxicity of Isuprel was determined in 16 healthy dogs of both sexes chosen from animals recently procured. The results obtained are presented in Table II. When a dose of 10 mg/kg was given by intubation, toxic symptoms began to appear. Two of the 3 animals at this dose level showed no symptoms other than depression and some apprehension, although the heart rate was increased as much as 164 beats/min. 15 minutes after the drug administration. The third animal vomited frequently and appeared restless for 2½ hours after receiving Isuprel. One death was observed at 15 mg/kg. Gross examination revealed an acute respiratory condition, but it seems improbable that this resulted directly from drug administration. Animals given doses of 20 mg/kg vomited frequently during the period 15 to 60 minutes after medication. This was followed by a period of depression lasting for more than 2 hours, with a return to normal in less than 24 hours. Of the 3 animals given

50 mg/kg, 2 showed less severe toxic symptoms than were observed on lower doses. The third animal showed, in addition to vomiting, restlessness and depression, a marked cardiac arrhythmia and profuse salivation. In 24 hours the dog appeared normal, but 4 days later was found dead in the cage. Pathological examination revealed petechial hemorrhages of the endocardium, extensive hemorrhages of the lungs with necrosis, acute bronchopneumonia and pulmonary abscess. Autolysis of the gastric mucosa was also noted.

Subcutaneous administration of 2-5 mg/kg caused salivation and restlessness followed by depression. After subcutaneous doses of 10-20 mg/kg, the symptoms were similar to those observed in dogs after oral doses of 20-50 mg/kg. Less than 2 hours after medication 1 animal given 15 mg/kg died. Pathological examination showed ecchymotic interstitial hemorrhages of the pancreas and congestion of the spleen and kidneys. The animal given 20 mg/kg was found dead 24 hours after drug administration. Gross examination revealed the same general findings as those described above.

A group of 18 rabbits weighing from 1.3-2.3 kg was given Isuprel by intravenous injection in doses of 35-60 mg/kg. Animals

TABLE II.
Acute Toxicity of Isuprel Hydrochloride in Dogs.

			Results
Dose, mg/kg	Time after drug, min.	Change in heart rate, beats/min.	Other observations
Subcut.			
2	5	+116	Salivating, nervous
	15	+100	Restless
	55	+122	Depressed
	210	+136	Vomited
	300	+108	Behavior normal
5	5	+ 86	Panting, salivating
	10	+ 82	Restless
	15	+ 78	Depressed
	55	+114	Depressed
	305	+128	Depressed
10	5		Vomited twice
	10	+174	Panting
	30	+150	Arrhythmia, panting, depressed
	105	+148	Arrhythmia, panting, depressed
	310	+190	Alert, panting
15	5	+154	Restless, panting, salivating
	10	+122	Arrhythmia, panting, salivating
	20	+100	Arrhythmia, depressed, diarrhea
	40	+116	Arrhythmia, profuse salivation (frothing), panting heavily
	70	+148	Salivating, panting, depressed
	100		Dead
20	10	+126	Panting, depressed
	15	+104	Arrhythmia, depressed
	45	+ 84	Arrhythmia, restless, apprehensive
	75	+108	Arrhythmia, restless, apprehensive
	150	+136	Depressed, breath short, almost inaudible gasps
	180	+152	Panting, apprehensive, slight salivation
	300	+ 92	Quiet
	(24 hr)		Dead
Oral			
10	5	+144	Depressed, apprehensive
	15	+164	Depressed, apprehensive
	65	+140	Depressed, apprehensive
	95	+100	Depressed, apprehensive
	(24 hr)	0	Apparently normal
15	5	+ 75	Restless
	15	+100	Restless, whining
	30	+100	Depressed
	60	+ 84	Very depressed
	(4 days)		Dead
20	5	+108	Skin hot and pink
	10	+142	Panting, coughing
	15	+112	Vomited twice, panting, apprehensive
	25		Vomited several times, panting heavily
	35	+128	Vomited twice, panting
	60	+136	Lying down, quiet, panting
	100	+140	Lying down, quiet
	(24 hr)	+ 16	Apparently normal
50	5	+ 84	Nervous, vomited
	10	+108	Restless
	20	+136	Vomited, restless
	40	+164	Marked cardiac arrhythmia, profuse salivation
	70	+140	Nervous, panting heavily
	115	+ 82	Lying down, alert, panting
	425	+ 70	Slightly depressed
	(24 hr)	0	Apparently normal
	(4 days)		Dead (1/2)

given 35-45 mg/kg showed general asthenia immediately following injection but seemed normal within 5-15 minutes. One of the animals given 35 mg/kg appeared normal at 60 minutes but at 65 minutes convulsed and died. At 30 minutes after medication both animals given 40 mg/kg and 1 of the 2 at 45 mg/kg convulsed and died. At onset of convulsions pallor was noted in the unpigmented area around the mouth and the scleral vessels of the eye became less distinct. At 50 mg/kg all animals showed general asthenia with some respiratory difficulty immediately following injection. Three of the 4 rabbits appeared normal 5 minutes later, but convulsed suddenly 1-1½ hours after injection. Of this group, only 1 animal recovered. The fourth rabbit suffered clonic spasms with opisthotonos within 5 minutes after injection followed by death in 7 minutes. Three of the 6 animals given 55 mg/kg died within 3 minutes after medication, with respiratory difficulty, clonic spasms and opisthotonos as prominent manifestations. Two animals appeared to recover from these symptoms after 10 minutes but were found dead at 8 and 48 hours, respectively, after medication. One animal recovered completely 15 minutes after injection. Both rabbits given 60 mg/kg showed the above symptoms with death at 10 minutes and 1½ hours, respectively.

Subacute Toxicity. A group of 20 rats weighing from 75-136 g and equally divided between the sexes were given 100 mg/kg of Isuprel by intubation daily for 15 days. Throughout the experiment all rats grew at a normal rate and showed no toxic symptoms. The animals were sacrificed at the end of the experiment for gross and histopathological examination. No significant abnormalities were found.

Eleven female rats were given subcutaneous injections of 100 mg/kg of Isuprel daily for 5 days. Six of this group were weanling rats weighing 48-60 g, and the remaining 5 were adults weighing 193-225 g. A similar group of rats was injected with distilled water as controls and 3 days after the final medication all rats were sacrificed. Although a gross examination of both experimental and control

rats showed enlarged uteri with prominent blood vessels, vaginal smears did not indicate estrus. It was therefore concluded that this condition must be normal for these rats.

Chronic Toxicity. Six dogs (3 male and 3 female), selected from animals recently procured, were dewormed and immunized in the usual manner. Isuprel was administered orally in a daily dose of 5 mg/kg for 6 to 17 weeks. At intervals of a week, chemical analyses for blood sugar, chloride, non-protein nitrogen and serum protein, and a determination of the formed elements of the blood were made. Throughout the experiment the blood picture remained normal. All animals appeared healthy and gained weight in the course of the test. Three and a half weeks after the beginning of drug administration 1 of the females delivered a litter of 5 normal pups. Gross and histopathological examination of the vital organs of all test animals revealed no significant abnormalities.

Isuprel was incorporated into a standard rat diet to make 0.5% by weight and this mixture was given as the only source of food to 30 weanling rats (15 male, 15 female) until the smallest animal weighed 146 g (6 weeks). A control group of 20 rats, litter mates of the above, was given the standard diet without Isuprel. There was no difference in the rate of growth between the experimental and control groups. Gross and histopathological examination disclosed no significant abnormalities.

Summary. Isuprel has a low acute toxicity in mice. Epinephrine is about 24 (intravenous) to 107 (intraperitoneal) times more toxic than this substance. Intravenous injection of Isuprel in rabbits was usually characterized by delayed deaths (30-90 minutes), the onset of terminal symptoms being rapid and unpredictable. Some deaths were observed at all doses tried (35-60 mg/kg). Subcutaneous injection into rats of 100 mg/kg once daily for 5 days caused no deaths.

Isuprel orally administered to dogs in doses of 10 mg/kg caused tachycardia, nervousness followed by depression and, with larger doses, salivation and vomiting. Death was observed with a dose as small as 15 mg/kg although

some animals survived oral doses of 50 mg/kg. Rats tolerated oral doses of 100 mg/kg daily for 15 days. Oral administration to dogs of 5 mg/kg daily for 6 to 17 weeks was well tolerated. Gross and histopathological examination of the vital organs of the medicated rats and dogs revealed no significant abnormalities. Isuprel did not interfere with growth

when incorporated into a standard diet to make 0.5% by weight and given as the only source of food to weanling rats.

From the above data it is concluded that Isuprel has a comparatively low toxicity.

The authors are indebted to Dr. J. O. Hoppe for the intravenous toxicity data on mice included in this communication.

16533

Radiation-Induced Hemorrhagic Disease in Chickens.*

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Several articles describing the hemorrhagic disease in animals and man following radiation have recently appeared.¹⁻³ In these subjects hemorrhage is one of the predominant features in the postradiation syndrome. Allen and Jacobson¹ concluded that, contrary to the prevalent idea, thrombocytopenia plays only a secondary role in producing hemorrhage following radiation in dogs. They felt that the most significant change was the presence in the blood of an increased amount of free heparin. Liebow² and Cronkite³ mentioned the presence of increased numbers of mast cells in tissues of victims of the atomic bombings, a finding of possible significance in view of the suggested relationship between mast cells and heparin production.

The blood changes and hemorrhagic disease we observed in chickens following continuous internal radiation from P³² administered subcutaneously were significantly different from those reported above in animals. In comparing the results of our work with the previous

work, 3 factors should be kept in mind. First, the radiation differed in two respects from the external radiation of the above experiments. Throughout our experiment, radiation by P³² was continuous in contrast with the brief interval of exposure above; while widespread throughout the body, the P³² did not give the uniformity of body radiation which results from external total body gamma radiation. Second, the platelets or thrombocytes of the chicken are nucleated cells therefore differing in origin and character from the non-nucleated mammalian forms. Third, compared to the mammals studied, the chicken is relatively resistant to radiation.⁴

Our course of P³² injections killed 25% of the 20 experimental chickens and made those surviving at the end of the 17-day observation period moribund. The course of the blood counts and the coagulation times as measured by the capillary tube method using venous blood are recorded on the accompanying graph. The surviving birds were killed after 17 days of radiation, and clot retraction and blood chemistry determinations were made. Gross and microscopic autopsy studies were also done.

Observations. RBC counts decreased stead-

* Work done under contract from Office of Naval Research.

¹ Allen, J. G., and Jacobson, L. O., *Science*, 1947, **105**, 388.

² Liebow, A. A., and Warren, S. (Abstract), *Am. J. Path.*, 1947, **23**, 888, 892.

³ Cronkite, E. P. (Abstract), *Am. J. Path.*, 1947, **23**, 891.

⁴ Warren, S. L., and Whipple, G. H., *J. Exp. Med.*, 1923, **38**, 741.

HEMATOLOGIC CHANGES DURING RADIATION

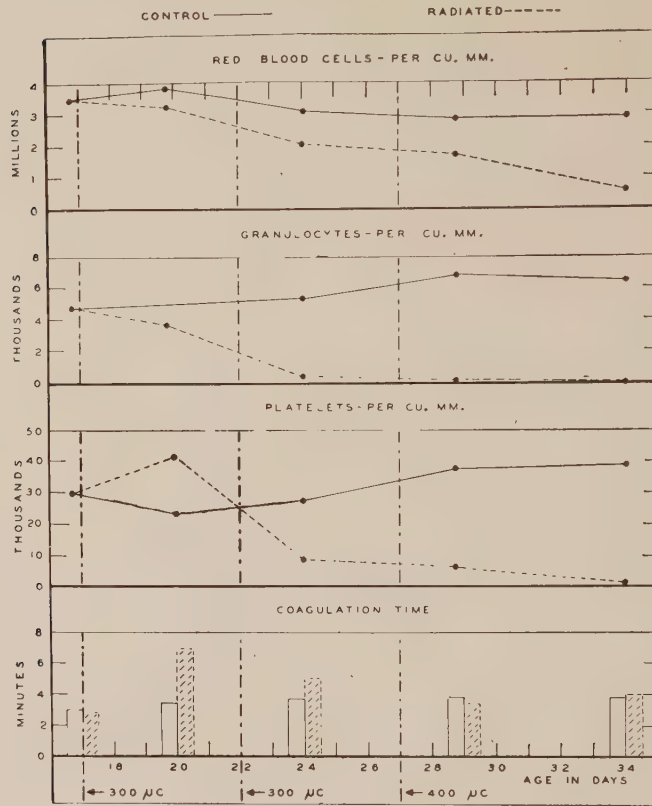


FIG. 1.

ily during radiation reaching 500,000 at the end of the 17-day observation period. Virtual agranulocytosis was reached after seven days of radiation and was maintained throughout the remainder of the experiment. The platelets showed a slight transitory rise and then fell off to insignificant levels after seven days and remained there. These changes in blood counts are comparable to the changes following total external body radiation.

The blood clotting time in both man and dog may be greatly prolonged or the blood rendered entirely incoagulable after radiation.¹ However, the radiated chickens had only a transitory increase in coagulation time over their control mates. This increase was of doubtful significance since the values were within the normal range for chickens reported by other workers.^{5,6} In the latter half of the experiment, when the radiation injury was

most severe, the coagulation time was not increased.

The character of the blood clot changed markedly in the radiated birds. After the mid-point of the observation period, when the platelets and granulocytes had reached very low levels, the blood clots were jelly-like in consistency and had completely lost the ability to retract. Even after ringing the clot and prolonged centrifuging very little serum could be separated from the clot.

Serum protein determinations, albumen globulin ratios, and blood cholesterol values were not altered in any of the radiated birds.

At autopsy these birds showed little hemorrhagic tendency relative to that described in mammals. Petechiae not exceeding 1 mm in

⁵ Wirth, *Folia haemat.*, 1942, **68**, 279.

⁶ Amendt, K., *Arch. f. d. ges. Physiol.*, 1922-1923, **197**, 556.

diameter were found scattered over the epicardium, thymus, and fascial planes of the muscles and tendons. Occasionally petechiae were found on the pleural surfaces of the lungs and on the peritoneal surfaces of the bowel. There were no large hemorrhages.

Microscopic study revealed the usual radiation injuries of the lymphoid tissue, bone marrow, and gonads, while other tissues showed little or no change. We failed to find any increase in the number of mast cells in our birds.

Conclusion. The thrombocytopenia developing after radiation and the associated poor clot and loss of clot retraction would seem to be the factors of importance in the hemorrhagic disease in chickens following P^{32} radiation. Since the blood coagulation time was not significantly altered even after severe radiation injury, it seems that the presence of

a heparin-like substance similar to that found in dogs would be unlikely.

Summary. In order to study the hemorrhagic disease resulting from radiation, we produced severe radiation injury by repeated subcutaneous injections of P^{32} into young chicks. Agranulocytosis and thrombocytopenia developed rapidly and during the same period the clotting mechanism of the blood was altered. However, there was no significant increase in coagulation time as found in animals and the hemorrhagic disease which developed was much less severe than that seen in animals. The increased number of mast cells associated with atomic bomb radiation injury in humans was not seen in the chickens.

The author is grateful to Miss Constance Sellman of the New England Deaconess Hospital and to Miss Harriett Kalison for technical assistance.

16534 P

Inulin Volume of Distribution as a Measure of Extracellular Fluid in Dog and Man.

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Current methods for measuring extracellular volume of water utilize substances which enter the tissue cells in a variable proportion (thiocyanate,¹⁻³ sodium,⁴ chloride,^{5,6} bro-

mid^{7,8}), are very rapidly excreted (sulfate,¹ sucrose¹), or are partially utilized by the organism (sucrose,⁹ mannitol^{10,11}).

Inulin has several advantages over any of the above substances: it is not metabolized to

* Fellow of the Institute of International Education and aided by the Dazian Fund.

[†] National Institute of Health Fellow.

[‡] Emanuel Libman Fellow.

¹ Lavietes, P. H., Bourdillon, J., and Klinghoffer, K. A., *J. Clin. Invest.*, 1936, **15**, 261.

² Gregersen, M. L., and Stewart, J. D., *Am. J. Physiol.*, 1939, **125**, 142.

³ Elkinton, J. R., and Taffel, M., *Am. J. Physiol.*, 1942-43, **138**, 126.

⁴ Kaltreider, N. L., Meneely, G. R., Allen, J. R., and Bale, W. F., *J. Exp. Med.*, 1941, **74**, 569.

⁵ Manery, J. F., *Am. J. Physiol.*, 1940, **129**, 417.

⁶ Amberson, W. R., Nash, T. P., Mulder, A. G., and Binns, D., *Am. J. Physiol.*, 1938, **122**, 224.

⁷ Wallace, G. B., and Brodie, B. B., *J. Pharm. and Exp. Therap.*, 1939, **65**, 214.

⁸ Weir, E. G., and Hastings, A. B., *J. Biol. Chem.*, 1939, **129**, 547.

⁹ Keith, N. M., and Power, M. H., *Am. J. Physiol.*, 1937, **120**, 203.

¹⁰ Smith, W. W., Finkelstein, N., and Smith, H. W., *J. Biol. Chem.*, 1940, **135**, 231.

¹¹ Dominguez, R., Corcoran, A. C., and Page, I. H., *J. Lab. and Clin. Med.*, 1947, **32**, 1192.

TABLE I.
Quantitative Urinary Recovery of Injected Inulin in Dog and Man.

		Amt of inulin inj. (mg)	Urine collection		
			Hrs after inj.	% of total amt recovered	% of total amt inj.
Dog	1	187.2	1.25	72	102
			2.75	94	
			4.5	100	
	2	186.0	1	70	102
			2.75	94	
			3.75	98.5	
			4.75	100	
Man	MG	5000	2.5	83	99
			3.5	90	
			4.5	93.6	
			6	96.5	
			7	97.7	
			10	99.8	
			12	100	
			19	100	
			30	100	
	ML	5000	2	77	102
			3	86	
			4	90	
			5	92.5	
			6	95	
			7	96.4	
			12	99.5	
			19	100	
			25	100	
			29	100	

any appreciable degree,^{12,13} and the circumstance that it is rapidly and quantitatively recovered in the urine after intravenous injection^{10,12} argues against storage in any tissue; it has a large molecular weight (ca.5101)¹⁴ and does not dissociate appreciably in solution,¹⁵ a circumstance which reduces the probability of penetration into cells; it does not penetrate the erythrocyte or escape through the normal renal tubules,^{13,16} it is physiologically inert when properly prepared^{12,17} and exerts negligible osmotic pressure. It possesses two disadvantages for this purpose: it diffuses slowly¹⁵ and it is rapidly excreted by glomerular filtration,^{12,13,18} these two circum-

stances rendering it unsuitable for use in the single injection method. The volume of distribution of inulin has been measured after ligation of both renal arteries,^{19,20} a procedure that permits equilibration but one that has obvious limitations.

The method here described permits the use of inulin for the measurement of that extracellular fluid in active interchange with the plasma water, provided that certain conditions are fulfilled: (1) constant extracellular fluid volume throughout the period of equilibration,

¹² Shannon, J. A., and Smith, H. W., *J. Clin. Invest.*, 1935, **14**, 393.

¹³ Smith, H. W., *Physiology of the Kidney*, Oxford University Press, New York, 1937.

¹⁴ Westfall, B. B., and Landis, E. M., *J. Biol. Chem.*, 1936, **116**, 727.

¹⁵ Bunim, J. J., Smith, W. W., and Smith, H. W., *J. Biol. Chem.*, 1937, **118**, 667.

¹⁶ Richards, A. N., Westfall, B. B., and Bott, P. A., *Proc. Soc. Exp. Biol. and Med.*, 1934, **32**, 73.

¹⁷ Smith, H. W., Chasis, H., and Ranges, H. A., *Proc. Soc. Exp. Biol. and Med.*, 1938, **37**, 726.

¹⁸ Shannon, J. A., *Am. J. Physiol.*, 1935, **112**, 405.

¹⁹ Kruhoffer, P., *Acta Physiol. Scand.*, 1946, **11**, 16.

²⁰ Kruhoffer, P., *Acta Physiol. Scand.*, 1946, **11**, 37.

TABLE II.
Volumes of Distribution of Inulin, Thiocyanate, Radioactive Sodium and Bromide in Dogs.

Exp. No.	Dog No.	Body wt, kg	Inulin			Volume of distribution Percentage of body weight			
			Duration of infusion, hr	Duration of collection, hr	Amt of inulin recovered, mg	% of total amt recovered	Inulin	Thiocyanate	Sodium Bromide
1	2	19.3	3.5	2 3 4	679.7 759.7 783.5	87 97 100	20.9		
2	2	18.0	2	2 4 5	327.8 360.6 361.9	90 99 100	21	32 31	
3	1	17.0	2	3 4 5.3	577.2 650.2 662.1	86 98 100	22.8	35.5	
4	1	16.4	3.3	2.8 4.5 5.5	563 624.9 635.1	88 98 100	23.2	34.3	31.4
5	3	15.0	3.3	1.8 6.8	163 202	81 100	21.4	32.5	26.7 28.1
6	3	15.0	3.8	1.8 2.5 4	116 161 167	70 96 100	21.4	30.7	
7	3	15.8	4	4 5.3	220 225	98 100	21.9	28.5	
8	4	13.5	3.3	3.3 5.3	177.2 182.1	97 100	21.4	25.8	33
9	4	13.8	5.8	1.3 4.8	149.5 193.3	77 100	21.6		32.7
10	5	12.5	2.8	0.5 5	97.3 192.6	50 100	20.3	31.2	30.5 31.5

TABLE III.
 Volume of Distribution of Inulin and Thiocyanate in Man.

Volume of Distribution of Inulin and Thiocyanate								
Exp. No.	Sub- ject	Body wt, kg	Inulin				Volume of distribution % of body wt	
			Duration of infusion, hr	Urine collection				
				Duration of collection, hr	Amt recovered, mg	% of total amt recovered	Inulin	Thiocyanate
1	MG	76.5	3.5	5.5	564	80	9.5	20.8
				10	698	100		
				19	698	100		
				24	698	100		
2	ML	74.0	6	.75	293	28	15.0	24
				3.5	805	78		
				5.3	922	89		
				15.3	1036	100		
3	IS	86.8	8	2	766	56	15.4	
				4	1049	77		
				13	1354	99		
				16.5	1365	100		
4	MG	77.0	20.5	9	1261	99	16.0	19.2
				17.5	1284	100		

(2) uniform distribution of inulin between all compartments of the extracellular space. To attain (2), a priming injection of inulin is followed by a constant rate of infusion adequate to compensate for the rate of excretion, the infusion being maintained for a period sufficient to insure uniform distribution. The minimal equilibration time is 1 to 2 hours in dogs and about 6 hours in man.[§]

Prior to the inulin injection a control blood and timed urine sample are collected for the determination of B_0 and U_0V , the latter being subtracted from the total urine inulin collected in the post-infusion period.

At the end of the infusion a blood sample is taken for determination of the plasma concentration of inulin. Simultaneously, the bladder is emptied by catheter and rinsed, and the infusion abruptly discontinued. The urine is then collected for 4 to 6 hours in dogs and 10 to 18 hours in man, which periods are necessary for excretion of at least 98% of the inulin in the body. In man urine collection was effected by spontaneous voiding. The quantity of inulin (in mg) recovered divided by the plasma concentration (in mg per cc)

[§] Renal function tests can be performed during this period.

equals the volume of distribution in cc.

In some cases thiocyanate, radioactive sodium (Na^{22} and Na^{24}) and bromide spaces were determined simultaneously by the single injection method. Blood samples for determinations of these substances were taken after 1 hour for thiocyanate, and after 3 hours for sodium and bromide. Chemical analyses were made by the method of Harrison for inulin,²¹ Crandall and Anderson for thiocyanate,²² and Friedman for bromide.²³ Radioactive sodium was measured with a Geiger-Müller counter.

Results and discussion. Complete recovery of a single injection of inulin in dogs is obtained in a period of 4 to 6 hours (Table I). In man similar recovery is obtained in about 10 hours (Table I), confirming the observations of others.^{10,13}

The volume of distribution of inulin as determined by the above method in dogs is shown in Table II. It ranges from 20.9 to 23.2% of the body weight (average 21.6).

²¹ Harrison, H. E., *Proc. Soc. Exp. Biol. and Med.*, 1942, **49**, 111.

²² Crandall, L. A., and Anderson, M. X., *Am. J. Digest. Dis. and Nutrition*, 1934-35, **1**, 126.

²³ Friedman, M. M., *J. Biol. Chem.*, 1942, **144**, 519.

This value is constant in the same animal in measurements made 15 to 30 days apart and with different periods of equilibration. These figures are substantially less than the volume of distribution of thiocyanate, bromide and radioactive sodium (Table II).

The volume of distribution of inulin in man (Table III) ranges from 15 to 16% of body weight and is not increased if the equilibration time is prolonged from 6 to 20 hours. This equilibration time was confirmed in a completely anuric patient whose inulin space after a single injection became constant after 6 hours. As in the dog, the simultaneous thio-

cyanate space was substantially larger than the inulin space.

Summary. The volume of distribution of inulin has been determined by a priming dose and a constant intravenous infusion to insure uniform distribution throughout the extracellular space, followed by collection of the total inulin excreted in the urine after the infusion is discontinued. The inulin space in the dogs ranges from 20.9 to 23.2% of the body weight (average 21.6) and in man from 15 to 16%, as compared with larger spaces obtained with thiocyanate, bromide and sodium.

16535

A Comparative Study of Blood Volume in Dogs.

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Although Evans Blue Dye (T-1824) is the most commonly used method for the determination of blood volume, there has been much controversy concerning the accuracy of the values obtained. While Gregersen¹ and others who have used the method for a long time uphold its validity, Hempden *et al.*² recently have offered experimental evidence to indicate inherent sources of error. The following experiments were carried out as part of a survey for a more accurate and rapid method of obtaining plasma and blood volumes.

In order to utilize constituents of the circulating blood which are known to remain in the vascular bed for relatively long periods, and to obtain a high degree of sensitivity, it was decided to use red cells and plasma protein tagged with radioactive elements. The red cells were labelled with P^{32} and the protein with I^{131} . Fe^{59} or Fe^{55} would undoubt-

edly be superior to P^{32} , but phosphorus has the advantage of permitting *in vitro* labelling without the necessity of metabolic incorporation into the cell. The methods for these studies were those of Hevesy,³ who first described the use of red cells tagged with P^{32} to study blood volume, and those of Fine and Seligman,⁴ who first reported blood volume studies employing plasma iodinated with I^{131} . Minor modifications were introduced by us.

The radioactive isotopes were obtained from the Isotope Division of the Atomic Energy Commission at Oak Ridge. Radioactive phosphorus has a half life of 14.3 days and emits a beta particle with a peak energy of 1.69 M.E.V. Radioactive iodine has a half life of 8.0 days. It emits a beta particle of .60 M.E.V. and two gamma rays of .367 and .08 M.E.V.

Healthy, full grown dogs weighing 9-22 kg were used. The tests were carried out in the

¹ Gregersen, M. I., *J. Lab. and Clin. Med.*, 1944, **29**, 12.

² Hempden, L., *et al.*, *Am. J. Physiol.*, 1947, **151**, 282.

³ Hevesy, G., and Zerahn, K., *Act. Physiol. Scand.*, 1942, **4**, 376.

⁴ Fine, J., and Seligman, A. M., *J. Clin. Invest.*, 1943, **22**, 285.

morning after an overnight fast of approximately 12 hours. Each animal was anesthetized with intravenous sodium pentobarbital, .44 cc/kg. No other medication was administered. The external jugular veins were used for injections of all test material and withdrawal of all blood samples.

Plasma volumes determined after the injection of T-1824 and iodinated plasma were done on the same day. Two or 3 days later the volumes were determined using red cells tagged with P^{32} .

Methods. T-1824—A control 5 cc blood sample was withdrawn into a Wintrobe hematocrit tube containing powdered heparin. The needle was left *in situ* and 5 cc of T-1824 injected. Blood was aspirated and reinjected 3 times in order to wash out any dye which adhered to the wall of the syringe. Exactly 10 minutes after injection of the dye, a second blood sample was withdrawn from the jugular vein on the opposite side. The plasma volume was determined using the Evelyn colorimeter. The specific gravities of the whole blood and plasma were determined by the copper sulfate method. The hematocrits and plasma protein values were obtained by reference to the charts provided by Phillips and co-workers.⁵

Iodinated plasma. The protein fraction of human plasma was activated with I^{131} according to the method described by Fine and Seligman.^{4*} Exactly 20 cc of iodinated plasma were injected. In the calculations no

* Preparation of I^{131} plasma:

1. A solution containing 5 mg of KI plus the desired amount of carrier free I^{131} and 30% HNO_3 were rapidly distilled into 25 cc of cold redistilled CCl_4 .

2. The CCl_4 solution of iodine was washed once with distilled H_2O and then added to 50 cc of chilled plasma which had previously been extracted with CCl_4 . 10 cc of 25% Na_2CO_3 were added and the mixture shaken until the color disappeared.

3. The plasma was then separated by centrifugation and dialyzed for 48 hours with cold running water.

4. The precipitated globulin was centrifuged down and the supernatant fluid was ready for use.

⁵ Phillips, R. A., *et al.*, *Bull. U. S. Army Med. Dept.*, 1943, **71**, 66.

correction was made for the injected plasma. Eight cc of blood were collected 5, 10, 15, and 20 minutes after the injection. Each sample was then centrifuged for 15 minutes at 3500 rpm. The activity of 1 cc of the plasma was determined with a Geiger-Müller counter. When the measurements on plasma were made without drying or ashing, immediate determination of the plasma volume was possible. (The standard and the samples were also counted after drying with no significant difference in the results.) The activity of 1 cc of the original iodinated plasma, diluted 1:100 was used as a standard. All samples were assayed using a bell shaped Geiger-Müller tube with a 3.0 mg per sq cm mica window. A conventional scale of 64 circuit was used. The 1 cc samples were placed in metal dishes of such dimensions that the surface area of the liquid counted was approximately 3.5 sq cm. The samples were counted at a 5 cm distance from the mica window. Each sample was assayed for that length of time necessary to reduce the counting error to less than 2%. The plasma volume was calculated as follows:

$$\frac{\text{Counts in Standard/cc} \times \text{Volume Injected} \times \text{Dilution of Standard}}{\text{Counts in Sample /cc}} = \text{Plasma Volume}$$

Radioactive red cells. Approximately 50 cc of heparinized dog blood, containing 5 microcuries of P^{32} were placed in a paraffin lined flask, and incubated for 2 hours at 37°C. The mixture was gently agitated every 5-15 minutes. Hevesy⁶ stated that these are the optimum conditions for maximum penetration of P^{32} into the red cell. At the end of the incubation period the blood was centrifuged at 2500 rpm for 15 minutes. The supernatant plasma was then decanted off. The packed red cells were washed once in normal saline and resuspended in sufficient normal plasma to restore the original volume. (In these experiments, a control blood sample was withdrawn from the animal for determination of residual activity from the iodinated plasma.) Then exactly 20 cc of the activated red cell

⁶ Hevesy, G., *et al.*, *Act. Med. Scand.*, 1943-44, **116**, 561.

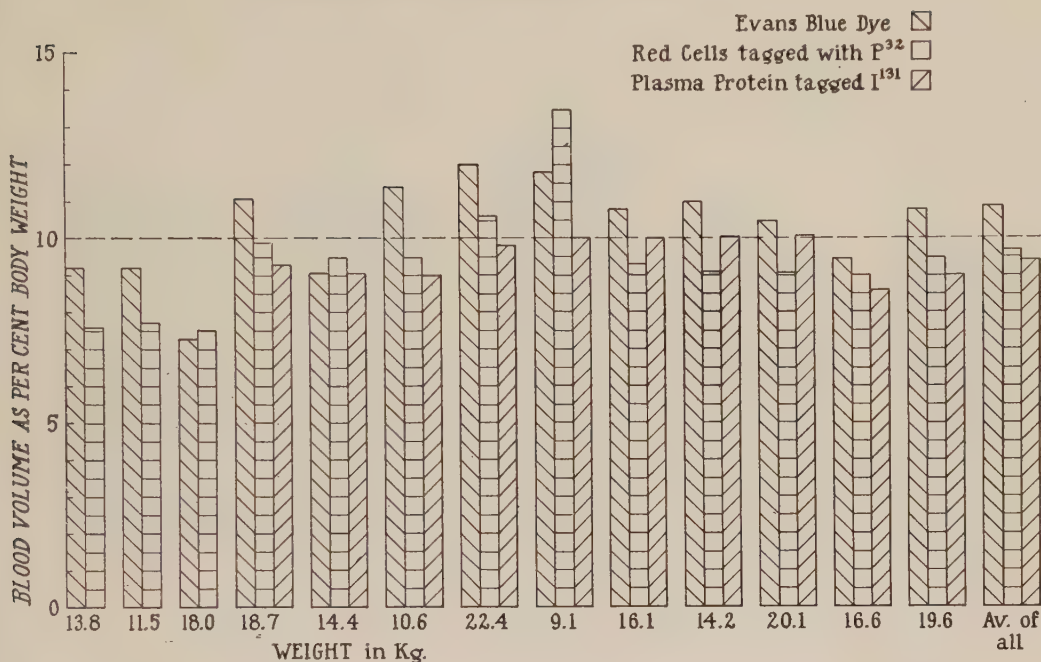
BLOOD VOLUME OBTAINED BY THREE METHODS
EXPRESSED AS PER CENT BODY WEIGHT.

FIG. 1.

Blood volume obtained by 3 methods expressed as per cent body weight.

suspension were injected. Five cc of blood were collected in a heparinized syringe 5, 10, 15, and 20 minutes after the injection. One cc samples were then counted for activity without drying. An aliquot of the original suspension was diluted 1:100 with 5% hydrochloric acid, and 1 cc of this was used as the standard. The blood volume was calculated as follows:

$$\frac{\text{Counts in Standard/cc} \times \text{Volume Injected} \times \text{Dilution of Standard}}{\text{Counts in Sample/cc}} = \text{Blood Volume}$$

Discussion. Blood volume studies by all the 3 methods described above were done on each of 10 dogs. P³² and T-1824 volume determinations were completed on 4 additional dogs. Using the plasma volume determined by iodinated protein and T-1824, and the jugular hematocrit, the total blood volume was calculated for each method. With the red cells tagged with P³², the dilution figures alone provided the total blood volume. In Fig. 1

the total blood volumes are shown in terms of per cent of body weight. From this chart it is seen that in most of the animals, the blood volume with T-1824 was larger than that obtained with the other 2 methods. The average blood volume (expressed as per cent of body weight) for the 10 animals using T-1824 was 10.5%; with P³², 9.7%; and I¹³¹, 9.4%.

The values obtained were all calculated using data from the 10 minute sample. After 10 minutes, T-1824 determinations err on the high side because of "dye leakage" from the vascular system. With the labelled red cell method, a sample taken earlier than 5 minutes does not allow adequate time for mixing, while after 10 minutes there is a "leakage" of P³² into the plasma and tissue fluids (Hevesy³). When iodinated protein is used, the blood samples can be drawn from 10 to 25 minutes following the injection with no evidence of significant decrease of iodinated protein in the plasma and thus no apparent change in the blood volume.

In Fig. 2 are examples of the variations in

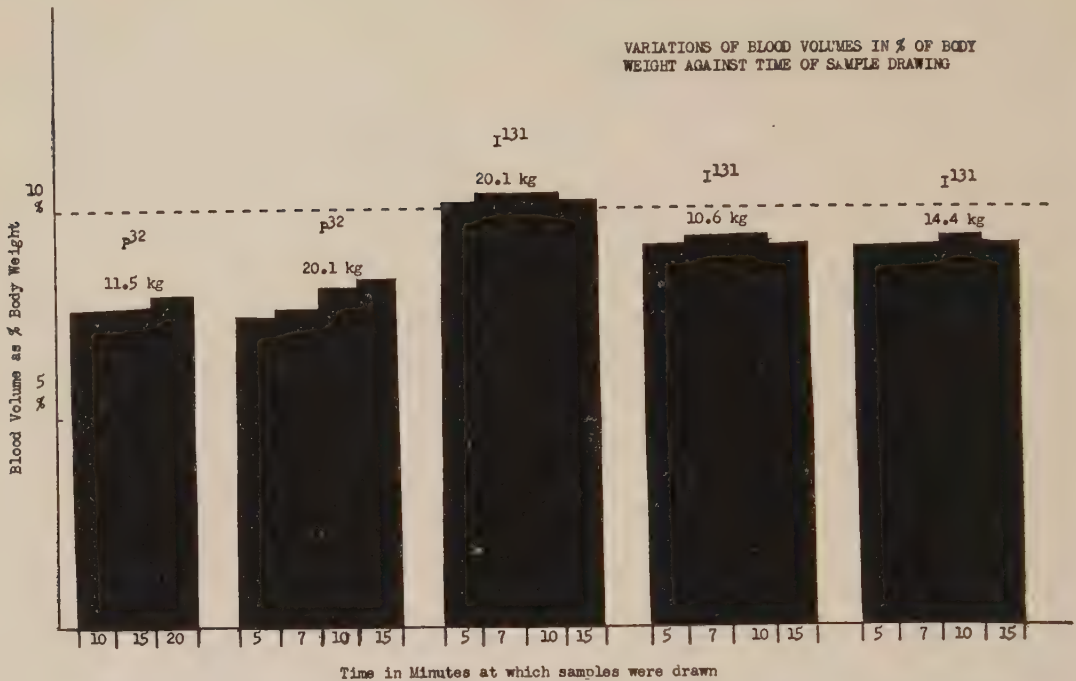


FIG. 2.

Variations of blood volumes in per cent of body weight against time of sample drawing.

blood volume (expressed as per cent body weight) with samples drawn at different time intervals. With the P³² method, an increase in volume is noted after 10 minutes, while with iodinated protein the blood volume remains more or less constant. All the animals were tested *in vivo* with a gamma ray counter for radioactivity over the thyroid gland without evidence of selective uptake of iodine.

Since plasma volumes determined with iodinated protein remain constant for a comparatively long period, this may be a method for studying rapid changes in effective circulating blood volume which occur under various circumstances such as vasodilatation and vasoconstriction after administration of drugs or during the course of surgical procedures.

The blood volume with red cells tagged with P³² is actually a measure of the dilution of red cells in the vascular system. If all the red cells under normal conditions are assumed to be in active circulation, the circulating total red cell volume can be calculated as the product of the (P³²) volume and the jugular hematocrit. In Fig. 3 the total cell volumes have

been calculated. The total blood volume may be calculated as the sum of total cell volume and the plasma volume. Table I shows a comparison of blood volumes when the calculated cell volume is added to the plasma volume determined by T-1824, and to plasma volume determined by iodinated protein. In Fig. 3, the blood volumes (expressed as per cent body weight) are compared when (a) cell volume determined by P³² is added to plasma volume estimated by I¹³¹, (b) plasma volume obtained with I¹³¹ and jugular hematocrit, (c) plasma volume obtained with T-1824 and jugular hematocrit. The volumes by the first two methods are of similar magnitudes.

Conclusions. 1. A comparison of 3 methods for the determination of blood volume was made on 10 dogs. 2. The average blood volumes expressed as per cent of body weight for the 3 methods were: T-1824, 10.5%; for red cells tagged with P³², 9.7%; and iodinated protein, 9.4%. 3. The iodinated protein method permitted accurate determinations of plasma volumes for a much longer period after injection than did the other two methods. This method was simple and direct. It per-

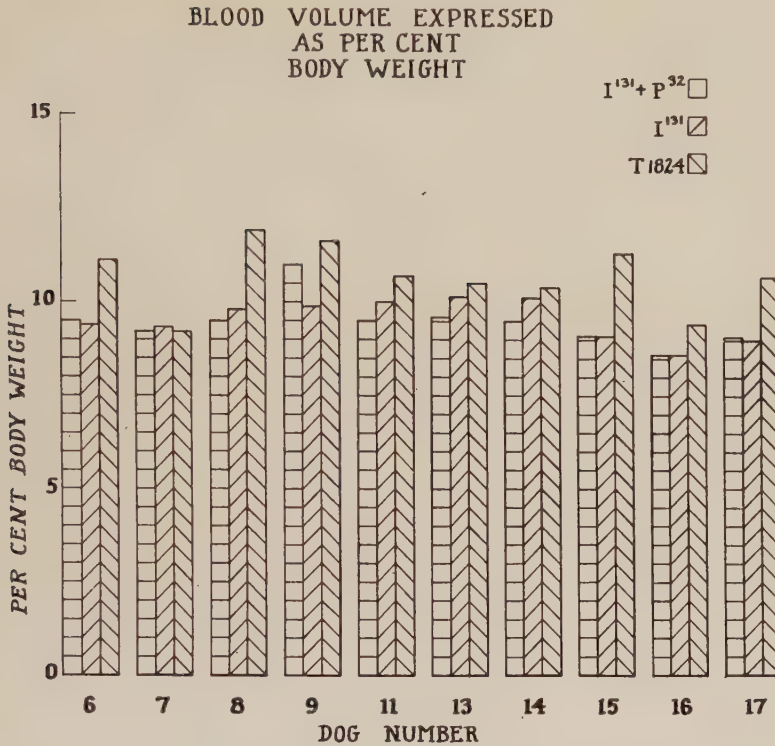


FIG. 3.
Blood volume expressed as per cent body weight.

TABLE I.
Comparison of Blood Volume Using Plasma Volume Obtained with T-1824 and I^{131} .

Dog	Wt, kg	Cell vol. P^{32} cc	Plasma vol. T-1824 cc	Blood vol. cc	Plasma vol. I^{131} cc	Blood vol. cc
6	18.7	801	1070	1871	986	1787
7	14.4	515	805	1320	811	1326
8	22.4	1015	1163	2178	1114	2129
9	9.1	590	534	1124	450	1040
11	16.1	668	928	1596	869	1537
13	14.2	550	911	1461	806	1356
14	20.1	908	1100	2008	1000	1908
15	10.6	415	648	1163	550	965
16	16.6	638	877	1515	795	1433
17	19.6	720	1296	2016	1056	1776

mitted calculation of the volume within a few minutes after the samples were obtained. It appeared to have a decided advantage over blood volume estimation with P^{32} impregnated red blood cells and the T-1824 dye method.

We wish to acknowledge the assistance given by Dr. Paul Lavik, Assistant Professor of Biochemistry, Western Reserve University, in preparing the iodinated protein.

Postganglionic Site of Action of Nicotine with Special Reference to its Direct Action on Blood Vessels.

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In the intact animal, administration of small amounts of nicotine is known to cause amongst other effects, constriction of the arteries of the ear and of the pads, a large rise of blood pressure, erection of hairs, dilatation of the pupil.¹ In an isolated organ, such as a strip of small intestine, nicotine induces inhibition of its movements, similar to those observed in the intact animal.²

These effects are attributed to the stimulating action of nicotine on the autonomic cells³ and/or to its action on the adrenal medulla, the latter resulting in augmented epinephrine secretion.⁴ In both instances, *i.e.* the intact animal and the isolated organs, it is assumed that nicotine acts only indirectly on the effector cells.

However, using the Laewen-Trendelenburg preparation in the frog, which is essentially an isolated vascular bed, we have previously reported that nicotine causes vasoconstriction.⁵ This fact is in agreement with that presented by other investigators.^{6,7} Since the presence of ganglion cells on peripheral blood vessels has been denied by most authors^{8,9} it appeared

to us that, in addition to its known stimulation of autonomic ganglion cells, nicotine may also have a more peripheral action. The present study was thus undertaken to investigate the postganglionic locus of action of nicotine with special reference to its direct action on blood vessels.

Methods and Material. The Laewen-Trendelenburg (L-T) preparation in frogs (*Rana pipiens*) was used as previously described.⁵ Frog Tyrode solution was used as a perfusate. Two Mariotte bottles were connected by a Y-piece with the perfusion system, *i.e.* the rubber tubing leading to the aortic cannula. One bottle contained plain Tyrode solution. The other contained, dissolved in the perfusate, one of the following substances: nicotine base (Eastman Kodak), curare (Intocostin, Squibb and Sons), tetraethylammonium hydrochloride (Etamon),[†] N,N, dibenzyl-beta-chloroethylamine hydrochloride (Dibenamine).[‡]

In addition to the "perfusion method", the effects of nicotine were also tested by the "bio-assay method" using the direct injection into the glass cannula inserted into the abdominal aorta. The vasomotor action of the different drugs was measured in terms of output variations of drops per minute.

Results. (A). *Effect of nicotine I.* *Injection of small amounts of nicotine: the bio-assay technic.* As previously reported, the injection of a small amount of nicotine (1-10 γ) in the L-T preparation elicits 2 major reactions: (a) Twitchings of the thigh and leg muscles, lasting as a rule from 30 to 60 seconds. (b) A marked reduction (50%-80%) of the outflow from the abdominal vein, as

* This investigation was aided by a grant from the Peripheral Vascular Disease Research Fund and the Elsa and William Menke Fellowship Fund.

¹ Langley, J. N., *J. Physiol.*, 1918-19, **52**, 247.

² Langley, J. N., and Magnus, R., *J. Physiol.*, 1905, **33**, 34.

³ Langley, J. N., *J. Physiol.*, 1901, **27**, 224.

⁴ Cannon, W. B., Aub, J. C., and Binger, C. A. L., *J. Pharm. and Exp. Therap.*, 1911-12, **3**, 379.

⁵ Haimovici, H., and Pick, E. P., *Proc. Soc. Exp. Biol. and Med.*, 1946, **62**, 234.

⁶ Handovski, H., and Pick, E. P., *Arch. f. Exp. Path. u. Pharmacol.*, 1913, **71**, 89.

⁷ Loewi, O., *J. Physiol. U. S. S. R.*, 1937, **22**, 390.

⁸ Woolard, H. H., *Heart*, 1926, **13**, 319.

⁹ Stoeck, P., Jr., *Mikrosk. Anat. d. veget. Nervensyst.*, Berlin, Springer, 1928, 67.

[†] Etamon was supplied by Parke, Davis and Co.

[‡] Dibenamine, Trademark of Givaudan-Delawanna, Inc. This drug was supplied by the Givaudan-Delawanna Co., Inc., Delawanna, N. J., through the courtesy of Dr. W. Gump.

expressed in terms of drops per minute. The duration of the vasoconstrictor effect varied, the average duration ranging between 15 and 30 minutes, until the output returned to its initial level. Nicotine tested several times in the same preparation induced regularly the same vasoconstrictor effect. Tachyphylaxis to nicotine was rarely noted. It was found, however, that some frogs exhibited individual variations as to the doses of nicotine and exceptionally some were entirely refractory to the usual small doses. In view of the latter fact, each individual preparation was first tested for its sensitivity to nicotine, before any further test was undertaken.

II) *Perfusion of the L-T preparation with nicotine.* 1. Continuous perfusion of the frogs vessels with a solution of nicotine in a concentration ranging from 1:250,000 to 1:50,000 induced: (a) Twitchings of the muscles lasting in most instances, several minutes. Exceptionally, twitchings were absent. (b) Marked vasoconstriction (50%-60%). The onset of the latter effect did not coincide with the start of the infusion. A delay of 2 to 4 minutes was noted. The maximum vasoconstriction appeared 5 to 10 minutes after the beginning of the nicotine perfusion. After 40 to 60 minutes the output returned, in most instances, to its initial level, despite continuous nicotine perfusion (Fig. 1). Additional injection of large amounts of nicotine (100 γ -500 γ)

remained practically without any effect. It is noteworthy that at this phase, the preparation still exhibited its usual sensitivity to the vasoconstrictor effect of epinephrine (0.5 γ to 1 γ). The self-blocking effect of nicotine, as observed in the prolonged perfusion of the L-T preparation, appears to be similar to the action of this alkaloid on other tissues. Indeed, nicotine in high concentrations produces a contracture of the rectus abdominis of the frog followed by relaxation, and then the muscle remains insensitive to further addition of nicotine.¹⁰ The paralysis of the tissues induced by high concentrations of nicotine seems to be a characteristic toxic manifestation of this alkaloid. 2. Perfusion for 3 to 10 minutes only, with concentrations of nicotine as above, induced identical patterns of vasoconstriction. In the same preparation, repeated perfusions with nicotine for short durations elicited the same degree of vasoconstriction. The self-blocking phenomenon of nicotine was thus obviated by reducing the perfusion time. The effects of nicotine in this instance were thus similar to those observed with the bio-assay technic.

III) Excision of both sympathetic chains and resection of all mixed nerves of the hind legs in the L-T preparation did not alter the action of nicotine on the blood vessels in both the bio-assay and the perfusion methods.

(B). *Effect of nicotine after tetraethylammonium hydrochloride (T.E.A.)* Because of the ability of T.E.A. ion to block the action of "nicotinic-stimulating" substances,^{11,12} it seemed desirable to test the effect of nicotine after its administration. T.E.A. was used in concentrations ranging from 1:10,000 to 1:25,000. Its perfusion through the frog's hind legs was not followed by any vasomotor effect *per se*. The vasoconstrictor effect of either nicotine or epinephrine remained unaltered by the perfusion with T.E.A. in preparations both with and without the sympathetic ganglia (Fig. 2). In perfusions with T.E.A.

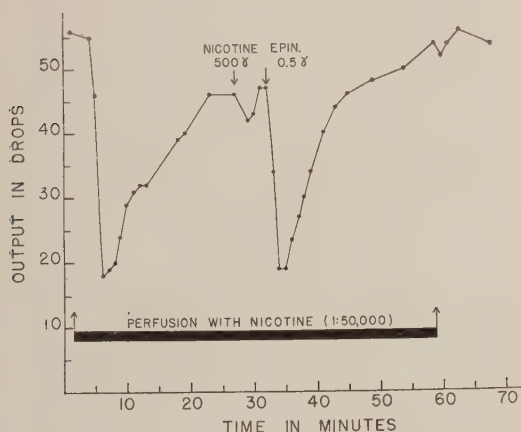


FIG. 1.

Self-blocking effect of nicotine. The L-T preparation becomes insensitive to additional large amounts of nicotine, while it retains its sensitivity to epinephrine.

¹⁰ Gasser, H. S., *Physiol. Rev.*, 1930, **10**, 35.

¹¹ Acheson, G. H., and Moe, G. K., *J. Pharm. and Exp. Therap.*, 1946, **87**, 220.

¹² Acheson, G. H., and Pereira, S. J., *J. Pharm. and Exp. Therap.*, 1946, **87**, 273.

lasting over an average of 30 minutes the vasoconstrictor effect of nicotine was slightly impaired. However, a larger dose of nicotine (*i.e.* 10 γ instead of 5 γ used in the control test) exhibited the same ability to induce marked vasoconstriction. Muscular twitchings usually induced by nicotine were abolished in most cases by T.E.A. but those induced by nerve stimulation remained unaffected by this agent.

(C). *Effect of nicotine after curare.* Langley stated that "in the frog the postganglionic nerves are not exempt from paralysis by curare as they are in the bird and the mammal."¹ It seemed then of interest to test the action of nicotine after administration of curare. Curare, as a d-tubocurarine chloride preparation (Intocostin, Squibb), was used in Tyrode solution in concentrations ranging from 1:12,500 to 1:50,000. The time of perfusion with curare varied from 3 minutes to over 1 hour. Curarization was considered satisfactory when stimulation of the mixed nerves of the hind legs failed to induce muscular twitchings.

The effects following injection of nicotine, during or after perfusion with curare, varied with the concentration of the latter and the duration of the perfusion. In high concentrations of curare (1:12,500) with time-perfusion

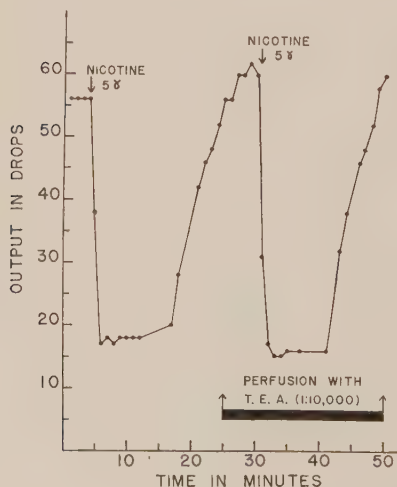


FIG. 2.

Unaltered effect of nicotine after tetraethylammonium hydrochloride in a L-T preparation without sympathetic ganglia.

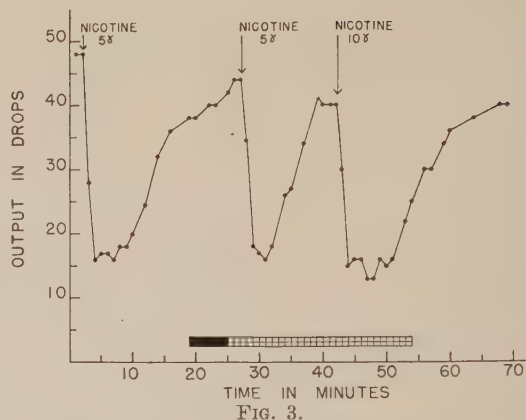


FIG. 3.

Unaltered effect of nicotine after curare. The plain bar represents the perfusion time with curare (Intocostin, 1:50,000). The cross-hatched bar represents the effective curarization time.

exceeding 10 minutes, nicotine induced only slight vasoconstriction. In lower concentrations (1:50,000) with time-perfusion not exceeding 3 to 10 minutes, nicotine exhibited almost, if not the same degree, of vasoconstriction as that obtained in the control tests (Fig. 3). Care was exercised to ascertain the curarization of the preparation throughout the testing of nicotine.

(D) *Effect of nicotine after Dibenamine.* Dibenamine being a potent and highly specific adrenergic-blocking agent,^{13,14} the study of the effect of nicotine on the blood vessels after blocking the transmission of sympathetic postganglionic impulses with this agent seemed of further interest. Dibenamine, in the concentration of 1:250,000 to 1:25,000 while inhibiting the vasoconstrictor action of epinephrine completely within 5 to 10 minutes after the onset of the perfusion, does not influence the action of nicotine. In addition, within 30 to 45 minutes after the onset of the perfusion, when the action of large amounts (50 γ -1000 γ) of epinephrine is completely blocked, nicotine still induces notable vasoconstriction and visible twitchings of the muscles.¹⁴

Discussion. From the foregoing data it appears that after excision of both sympathetic

¹³ Nickerson, M., and Goodman, L. S., *J. Pharm. and Exp. Therap.*, 1947, **89**, 167.

¹⁴ Haimovici, H., *Proc. Soc. Exp. Biol. and Med.*, 1947, **64**, 486.

chains and resection of all spinal nerves of the frog's hind legs in the L-T preparation, nicotine induces the same degree of vasoconstriction as before. This seems to indicate that in this preparation the locus of action of nicotine is not in the autonomic ganglia but more peripheral. In order to test this interpretation it remained to demonstrate that the hind legs of the frog were ganglion-cell free. Both pharmacologic and histologic methods were used to that end.

The T.E.A. ion is known to be capable of blocking the stimulating action upon ganglion cells by "nicotinic-stimulating" substances.^{15,16} Since the perfusion of the hind legs with this agent did not abolish the nicotinic vasoconstriction it appears that nicotine acts beyond the ganglion cells.

The action of nicotine was further investigated after blocking the postganglionic sympathetic transmission with curare and Dibenamine. Curare, in the frog, unlike in the birds and mammals, extends its action to postganglionic autonomic fibers. From the above data it appears that despite effective curarization, nicotine still continued to exhibit its vasoconstrictor action.

With Dibenamine, an adrenergic-blocking agent, similar observations were made. It is of interest to note that De Vleeschhouwer¹⁷ reported "that 10 to 15 mg/kg Dibenamine do not reverse the hypertensive action of nicotine in the atropinized dog. Larger quantities decrease slightly, but never reverse, the vasopressor effects of nicotine." The observations made in the intact animal seem to confirm our own observations made in the L-T preparation that nicotine is also capable of acting beyond the postganglionic apparatus of the autonomic nervous system.

The ability of nicotine to act directly on blood vessels without synaptic intervention is shown by the fact that it also induces vasoconstriction in the isolated rabbit's ear.¹⁸

In man, Coon and Rothman^{19,20} injected nicotine intradermally and noted pilomotor activity and sweating. These authors believe that nicotine in this instance acted through an axon reflex mechanism within the skin. In the light of data presented in this study there is reason, however, to believe that the locus of nicotine action was more directly on the neuroeffector cells.

The validity of the interpretation of the above pharmacologic findings is further supported by our histologic study of the blood vessels of the frog's hind legs. Numerous serial sections of sciatic, femoral and tibial vessels and the corresponding nerves of 10 hind legs were studied. No ganglion cells were encountered.⁹ These findings in the frog are thus in agreement with previous reports concerning mammalian blood vessels of the extremities.^{8,9} It is of interest to mention, however, the presence of abundant perivascular melanophore cells in the frog. The physiologic significance of the latter is not clear as yet. There are no known connections between nerve fibers and the chromatophores. As is well known, the pituitary secretion exercises a direct effect upon these pigment cells. Shen²¹ has shown that while nicotine provokes an intense and prolonged melanophore-expanding action upon normal pale frogs, it fails to do so in hypophysectomized frogs or in isolated legs. It appears, therefore, that the vasoconstrictor action of nicotine observed in the L-T preparation is quite unrelated to the perivascular melanophore cells.

Summary and Conclusion. In the L-T preparation, which is essentially an isolated vascular bed, nicotine induces marked vasoconstriction. Resection of both sympathetic chains and of all spinal nerves does not alter the action of nicotine. T.E.A. ion, capable of blocking the action of the "nicotinic-stimulating" substances upon ganglion cells, curare,

¹⁸ Pick, E. P., Personal communication.

¹⁹ Coon, J. M., and Rothman, S., *J. Pharm. and Exp. Therap.*, 1940, **68**, 301.

²⁰ Coon, J. M., and Rothman, S., *J. Pharm. and Exp. Therap.*, 1941, **73**, 1.

§ I am indebted to Dr. Frederick G. Zak for his help in this study.

²¹ Shen, T. C. R., *J. Physiol.*, 1937, **90**, 518.

¹⁵ Burn, J. H., and Dale, H. H., *J. Pharm. and Exp. Therap.*, 1914, **6**, 417.

¹⁶ Hunt, R., *J. Pharm. and Exp. Therap.*, 1926, **28**, 367.

¹⁷ De Vleeschhouwer, G. R., *Proc. Soc. Exp. Biol. and Med.*, 1947, **66**, 151.

capable of paralyzing postganglionic fibers, and Dibenamine capable of blocking the same fibers, do not abolish the vasoconstrictor action of nicotine. Serial sections of the vessels of the frog's hind legs have shown the absence of ganglion cells.

It appears, therefore, that in the L-T preparation the site of action of nicotine is

peripheral to the postganglionic fibers, possibly directly on the blood vessels. In the intact animal, it may be assumed that in addition to its known sites of action on ganglion cells, and adrenal medulla, nicotine may also act directly on the neuroeffector cells of the blood vessels.

16537 P

Effects of Intrathecal Administration of Epinephrine on Cardiac Rhythm in Dogs Anesthetized with Cyclopropane.

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Recently there has been a trend in clinical anesthesia to combine epinephrine with local anesthetic drugs to prolong the duration of spinal anesthesia. At times it is necessary to supplement spinal anesthesia with a rapidly acting drug such as cyclopropane. It is well established that cyclopropane increases cardiac irritability and this increased irritability is further enhanced by epinephrine. In dogs, as little as .01 mg of epinephrine per kilo intravenously precipitates ventricular premature beats, tachycardia or fibrillation during deep cyclopropane anesthesia. There is some evidence that vasopressors do not quickly pass from the subarachnoid space into the blood stream. Bray, Katz, and Adriani¹ have shown that epinephrine, neosynephrine, ephedrine and oenethyl cause no vasopressor response when administered intrathecally in therapeutic doses in man. Inasmuch as so little epinephrine causes serious circulatory changes, it is important to know whether or not the passage of epinephrine from the subarachnoid space into the general circulation is sufficiently rapid to be a hazard under such circumstances.

Method. Observations were made on 6 dogs. Thirty mg of nembutal per kilo was administered intraperitoneally to facilitate the

insertion of an endotracheal tube. Cyclopropane and oxygen were administered by the carbon dioxide absorption technique to the point of intercostal paralysis and maintained at this level. After 20 minutes of cyclopropane anesthesia control electrocardiograms were obtained using lead II.

The first dog was given the standard test dose of .01 mg kilo body weight in 5 cc normal saline intravenously until ventricular tachycardia occurred. Meek² *et al.* have found that dose produces reflex vagal inhibition of the pacemaker with or without escape of the A-V node bundle or ventricle but without tachycardia or fibrillation. With cyclopropane this same dose causes ventricular premature beats, tachycardia or fibrillation. After the rhythm had returned to normal 1 mg of epinephrine was administered intrathecally. One milligram is the upper limit of the dose usually employed clinically. Continuous electrographic recordings were made prior, during and immediately after the injection. The dog was observed for 1 hour, at the end of which time the standard test dose of epinephrine was repeated intravenously. The second dog was prepared as the first except that at the end of 1 and 2 hours 2 mg and 5 mg of epinephrine

¹ Bray, K., Katz, S., and Adriani, J., in press.

² Meek, W. J., Hathaway, H. R., and Orth, O. S., *J. Pharm. and Exp. Therap.*, 1937, **61**, 240.

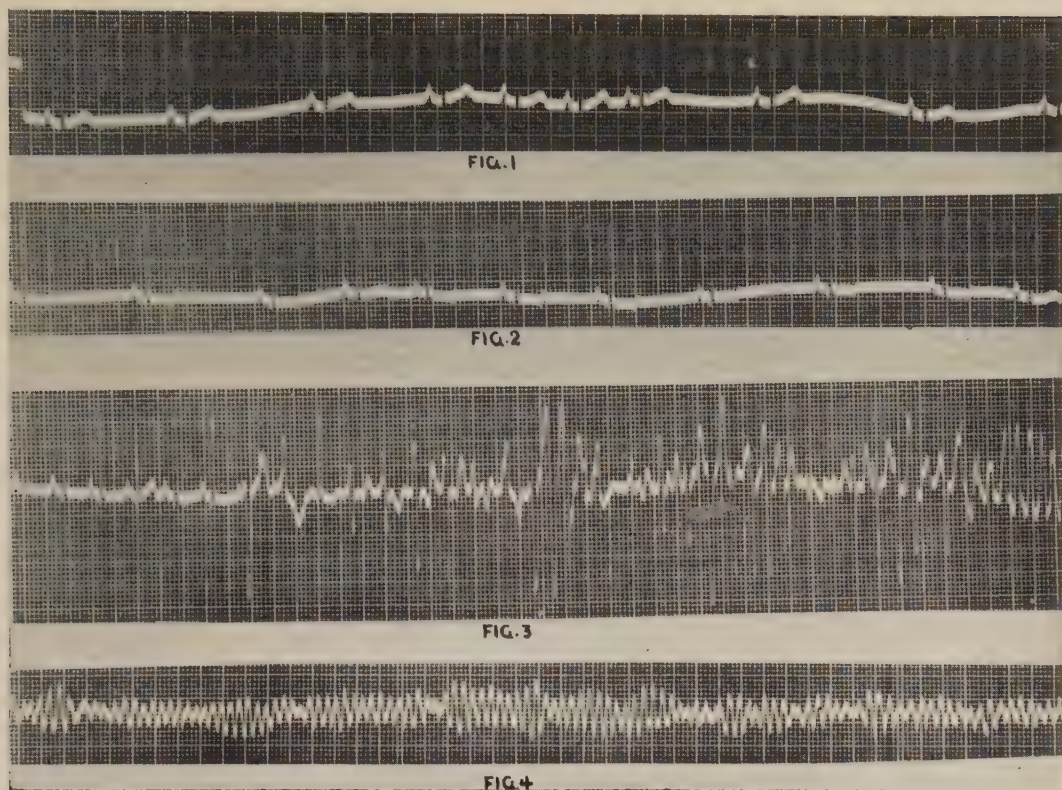


FIG. 1. Electrocardiogram. Control after nembutal and cyclopropane anesthesia.
FIG. 2. After 1 cc epinephrine intrathecally during cyclopropane anesthesia.
FIG. 3. After $\frac{1}{2}$ cc spinal fluid intravenously (withdrawn one hour after epinephrine injection). Ventricular premature beats proceeding to ventricular tachycardia.
FIG. 4. Ventricular fibrillation resulting from injection of spinal fluid.

respectively were administered intrathecally. At the end of the third hour the standard test dose of epinephrine was administered intravenously. The last 3 dogs were initially given 1 mg of epinephrine intrathecally but not the intravenous standard test dose. At the end of one hour the third dog received the standard test dose intravenously. Spinal fluid was withdrawn from the fourth and fifth dogs after one hour and 0.5 cc undiluted injected intravenously. Cardiac rhythm was consistently observed on the electrocardiograph prior, during and immediately after each intrathecal or intravenous injection.

Results. In all dogs ventricular tachycardia appeared after the intravenous test dose of epinephrine. When 1 mg of epinephrine was given intrathecally no change in cardiac rhythm was noted during the one hour period in any of the dogs during cyclopropane anes-

thesia. In dog 2 no changes in cardiac rhythm were noted after the second hour after 2 mg of epinephrine were administered intrathecally. During the third hour the 5 mg dose intrathecally produced paroxysms of ventricular tachycardia 88 seconds after injection. Rhythm returned to normal in 3 minutes. Although in all dogs 1 mg intrathecally caused no change in rhythm over a period of 1 hour, the standard test dose caused ventricular premature beats, tachycardia and fibrillation when given intravenously at the end of the hour. Dog 1 died from ventricular fibrillation. Spinal fluid withdrawn and given intravenously 1 hour after 1 mg of epinephrine had been introduced into the subarachnoid space apparently still contained epinephrine. One half cubic centimeter undiluted, given intravenously caused ventricular tachycardia to develop in 18.2 seconds in dog 4 and in 11.8

seconds in dog 5. Dog 5 then developed ventricular fibrillation and died. In order to observe what might occur if epinephrine is inadvertently injected into the interspinous ligaments instead of intrathecally, 1 mg was injected at this site in dog 6. A-V nodal rhythm, ventricular premature beats appeared 30 seconds later.

Summary and Conclusions. Epinephrine was administered intrathecally to 5 dogs anesthetized with cyclopropane. The characteristic changes which occur after intravenous epin-

ephrine is given did not occur. At the end of one hour epinephrine was still present in sufficient quantities in the spinal fluid to cause disturbances in cardiac rhythm when the spinal fluid was withdrawn and given intravenously. Presumably the passage of epinephrine from the intrathecal space into the blood stream does not occur or occurs so slowly that it is of little consequence in dogs anesthetized with cyclopropane. The same is not true if epinephrine is given intravenously or into the interspinous tissues.

16538

Further Studies on Nutritional Requirements of the Cotton Rat. (*Sigmodon hispidus hispidus*).*

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Previous studies¹ showed that the cotton rat (*Sigmodon hispidus hispidus*) requires thiamin, riboflavin, pantothenic acid, vitamin B₆, nicotinic acid and inositol in the diet for optimum growth. Since these animals are very susceptible to dental caries^{2,3} and are also susceptible to virus infections⁴⁻⁶ it was of

interest to extend the results obtained on their nutritional requirements. Studies have been carried out on the rate of growth obtained when diets low in choline, biotin, folic acid or nicotinic acid were fed. Preliminary studies on the reproduction performance have been conducted with the use of purified diets with and without the addition of liver extract.

Experimental—Care of Animals. Weanling cotton rats, 15-25 g in weight, obtained from our stock colony, were used in the growth studies. Litter mates and males and females were distributed as uniformly as possible among the groups within each experimental series. The animals were housed singly in screened bottom cages and food and water were provided *ad libitum*. The rate of growth for the different groups was observed over a period of 4-6 weeks.

In the reproduction studies one male and 1 or 2 females were housed in a colony cage with the screened bottom removed. Wood shavings and a metal can were included in each cage. The animals were placed together at an early age, usually before they were 6

* This work was supported in part by a grant from the United States Public Health Service. Acknowledgments are made to Helen Keene and Juanita Pou for valuable technical assistance and to Wilson Laboratories, Chicago, Illinois, for generous supplies of 1:20 liver extract.

† Present address: Division of Biochemistry and Nutrition, American Meat Institute Foundation, University of Chicago.

¹ McIntire, J. M., Schweigert, B. S., and Elvehjem, C. A., *J. Nutrition*, 1944, **27**, 1.

² Shaw, J. H., Schweigert, B. S., McIntire, J. M., Elvehjem, C. A., and Phillips, P. H., *J. Nutrition*, 1944, **28**, 333.

³ Shaw, J. H., Schweigert, B. S., Elvehjem, C. A., and Phillips, P. H., *J. Dental Research*, 1944, **23**, 417.

⁴ Armstrong, C., *U. S. Public Health Repts.*, 1939, **55**, 1719.

⁵ Jungeblut, C. W., *Proc. Soc. Exp. Biol. and Med.*, 1940, **43**, 479.

⁶ Armstrong, C., *J. Bact.*, 1940, **39**, 63.

weeks of age, since when adult animals are placed together, a high mortality often results. The animals usually remain in the can during the day, a factor which reduces the excitability of the animals considerably. Examinations were made for litters and the number of young counted, but the litter was disturbed as little as possible. The male was left with the female and several pairs have consistently produced a litter every month. The young were weaned at 14-17 days of age, sexed and weighed. Data were obtained, therefore, on the number of young born, number weaned, weight of young at weaning and the number of litters per female. Greater difficulties are inherent in obtaining data on reproduction as complete as can be obtained with the white rat. Discussions of methods of care and handling of cotton rat colonies have been reported.^{1,7,8}

Composition of Diets. The basal ration used in the growth studies was composed of the following: (in %) sucrose 67, purified casein 24, mineral mixture 4, corn oil 4.7, vitamin A & D conc. 0.3 and the following B vitamins (per 100 g) thiamin 250 μ g, riboflavin 300 μ g, pyridoxine 250 μ g, Ca pantothenate 2 mg, nicotinic acid 2.5 mg, choline 100 mg, inositol 100 mg, *p*-aminobenzoic acid 30 mg, biotin 10 μ g and pteroylglutamic acid 200 μ g. The vitamin deficient diets were prepared by omitting the appropriate vitamin from the B vitamin mixture.

This ration was modified for the reproduction studies by increasing the amount of casein to 30% and the vitamin A & D concentrate to 0.6% at the expense of the sucrose. The amounts of the B vitamins added were doubled with the exception of nicotinic acid, choline, inositol and *p*-aminobenzoic acid. For some groups 4% of 1:20 liver extract was added at the expense of the entire ration.

Results and Discussion. Growth studies. A severe choline deficiency could not be consistently produced either when the basal diet without added choline previously described

was fed or when a peanut meal ration^{9,10} low in choline and methionine was used. When animals with an initial weight of 15 g were used, more failed to survive than when larger animals were used. A total of 5 animals out of 24 fed the deficient diets died within the first 2 weeks on experiment. The average growth rates of the surviving animals were somewhat lower than for the groups fed 100 mg of choline chloride per 100 g of ration in addition to the basal ration. The variation was rather great, and although the results did not warrant extensive studies on the quantitative requirements, they appear to be qualitatively similar to those obtained with the white rat.

In previous studies¹ crystalline biotin and pteroylglutamic acid were not available. The present studies showed that the omission of either or both these vitamins did not appreciably affect the rate of growth. Eight to 19 animals were used in each group and the average rate of growth per week obtained when both vitamins were included was 10.0 g, biotin omitted 9.7 g, pteroylglutamic acid omitted 9.2 g and both omitted 9.4 g. Therefore it is concluded that dietary supplements of these factors are not needed when tested with the purified diet described. In accord with previous work^{1,11} the addition of liver extract to the ration increased the rate of growth.

Other findings revealed that dietary tryptophan was an effective precursor of nicotinic acid for the cotton rat as well as other animals.^{12,13} Consequently, studies were conducted to determine the effect of the amount of casein (tryptophan) in the diet on the dietary requirement of nicotinic acid. From the results shown in Table I, it can be seen

⁹ Engel, R. W., and Salmon, W. D., *J. Nutrition*, 1941, **22**, 109.

¹⁰ McKibbin, J. M., Thayer, S., and Stare, F. J., *J. Lab. Clin. Med.*, 1944, **29**, 1109.

¹¹ Schweigert, B. S., Shaw, J. H., Phillips, P. H., and Elvehjem, C. A., *J. Nutrition*, 1945, **29**, 405.

¹² Schweigert, B. S., Pearson, P. B., and Wilkening, M. C., *Arch. Biochem.*, 1947, **12**, 139.

¹³ Schweigert, B. S., and Pearson, P. B., *J. Biol. Chem.*, 1948, **172**, 485.

⁷ Meyer, D. B., and Marsh, M., *Am. J. Pub. Health*, 1943, **33**, 697.

⁸ Schweigert, B. S., *Vitamins and Hormones*, 1948, in press.

TABLE I.
Effect of the Level of Casein in the Diet on the Growth Response With and Without Nicotinic Acid Supplementation.

Level of casein fed %	Rate of gain*		Comments on group receiving the —N. A. diets
	—N. A. g/wk	+N. A.	
12	1.7 (10)	5.6 (7)	5 dead at 4 wks
18	5.7 (16)	7.1 (12)	4 " " 6 "
24	8.7 (7)	8.0 (6)	0 " " 6 "

* The supplemented groups received 1.0 mg nicotinic acid/100 g ration. The number of animals in each group is given in parentheses.

TABLE II.
Reproduction Studies.

Dietary regimen	No. of pairs	Total No. of litters	Avg No. young per litter	Avg wt of young and age at weaning*
Stock†	5/6‡	25	4.5	21.5 (14.6)
Basal	2/5	2	2.0	20.0 (14)
Basal — biotin or pteroylglutamic acid	2/11	2	1.0	None weaned
Basal + 4% 1:20 liver extract	5/7	17	2.8	22.1 (14.9)

* The average age at weaning is given in parentheses.

† Rockland rat pellets, complete.

‡ No. of pairs producing young/total No. of pairs in the group.

that the response due to nicotinic acid supplementation decreased as the casein level was increased. In previous work an 18% casein diet was used, and although nicotinic acid was again shown to increase the rate of growth, this effect disappeared when 24% casein was fed.

Reproduction Studies. Some of the animals that were used in studying the effect of ingesting diets with and without the addition of biotin and pteroylglutamic acid on the rate of growth were used for reproduction studies. One male and one female were placed in colony cages after 6 weeks on experiment and fed the basal diet described for reproduction studies with the appropriate vitamin omitted. The reproduction performance of these animals was compared with that for pairs that received stock ration or the basal ration + 4% 1:20 liver extract. These tests have been conducted over a 9 months period and are summarized in Table II. Data obtained on animals from pairs that died within the first 6 months of this period were not considered in these tabulations. It can be seen that the purified diets which contained pteroylglutamic acid and biotin or only one of these supplements failed to support successful reproduc-

tion. Only 4 pairs of a total of 16 produced young, and each of these pairs only produced one litter which was also small in size. Since inadequate reproduction was obtained when both pteroylglutamic acid and biotin were included, the data obtained do not permit an accurate evaluation of the essentiality of either biotin or pteroylglutamic acid.

The addition of 4% of 1:20 liver extract to the basal diet did improve the reproduction performance, however. Five of a total of 7 pair produced young and 2 of the pairs have produced 5 and 6 successive litters when this diet was fed. The litters were smaller in size, however, than those that were obtained from pairs fed stock ration. These data suggest, therefore, that liver extract contains factors which are necessary for reproduction in the cotton rat, but that the size of the litters is still not optimal. It is possible that the addition of cellulose to the purified rations may be beneficial since Howell and associates¹⁴ have recently demonstrated that the addition of 10% cellulose to a purified diet prevented trichobezoar in the cotton rat and

¹⁴ Howell, S. R., Schlaack, C. A., McCay, C. M., and Taylor, B. L., *Science*, 1948, **107**, 424.

increased the rate of gain. A variable accumulation of hair in the stomach was also observed in the present study for stock pairs fed purified diets. Further work is needed to determine the value of adding cellulose to the ration on the reproduction performance.

The weight of the young at weaning for both sexes was similar, *i.e.* 20.5 for 32 males and 21.0 for 44 females from a pair fed stock ration. It can be seen from Table II that the weaning weights of young were similar for the groups receiving different diets. This observation cannot be accurately evaluated, however, since smaller litters were weaned by the groups fed purified diets, and thus would result in a variable stress of lactation.

It is recognized that the purified diets used favor the development of dental caries and this may have affected the reproduction performance. The animals ate the rations readily, however, and reached a mature weight ap-

proximating that obtained when stock ration was fed. A few pairs were fed starch as the carbohydrate, but their performance did not appear to be improved over that for the groups fed sucrose diets.

Summary. The cotton rat (*Sigmodon hispidus hispidus*) was shown not to require a dietary source of biotin or pteroylglutamic acid for optimum growth when a purified ration was fed. The growth response obtained with nicotinic acid supplementation was dependent on the casein (tryptophan) content of the diet in that as the casein level was increased the growth response attributable to the addition of nicotinic acid was decreased. A poor reproduction performance was observed when purified diets were fed. Improvement was noted when liver extract was fed in addition to the purified diet or when stock ration was fed.

16539

Pyridoxine, Choline and *l*-Methionine Content of Parenteral Liver Extract.

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In the nonspecific use of parenteral liver extract, the physician is not much interested in a statement of antianemia potency as he is in knowing the amounts of the several biologically active substances (factors of the B complex) present. Numerous requests for such information have made it necessary to analyze the various types of liver extract. The advent of the several microbiological assays made it possible to determine the amounts of several of the B factors. A previous article¹ contains data for total solids, total nitrogen, riboflavin, niacin, pantothenic acid, and folic acid (*L. casei factor*) present in several samples of refined, concentrated extracts and in the dilute, crude preparations. In this brief paper, data is presented for the pyridoxine, choline, and *l*-methionine content of twelve

different commercial extracts.

Materials and Methods. The liver extracts, conforming to U.S.P. standards, included in this report represent random samples purchased from a wholesale drug-gist and unselected samples of our own products. The total amounts of pyridoxine, choline, and *l*-methionine extracted from whole liver by hot, acidified water (pH 5.0–85°C) are shown, numbers 34 and 35 in Table I. The method of Hochberg, Melnick, and Oser² has been used for the determination of pyridoxine. The microbiological method described by Stokes, *et al.*³ has been used for the estimation of *l*-methionine. Choline has

² Hochberg, M., Melnick, D., and Oser, B. L., *J. Biol. Chem.*, 1944, **155**, 119.

³ Stokes, J. L., Gunness, M., Dwyer, I. M., and Coswell, M. C., *J. Biol. Chem.*, 1945, **160**, 35.

¹ Clark, G. W., *Am. J. Med. Sci.*, 1945, **209**, 520.

TABLE I.
Vitamin Content of Liver Extracts for Parenteral Use II. A Comparison of Crude and Concentrated Preparations.

Extract No.	U.S.P. units per cc	Pyridoxine HCl		Methionine HCl		Choline chloride	
		Per cc, μ g	Per unit, μ g	Per cc, mg	Per unit, μ g	Per cc, mg	Per unit, μ g
22	15	7.9	0.5	3.1	207	8.9	593
23	15	3.4	0.2	3.4	227	7.0	467
24	15	21.0	1.4	1.2	80	1.1	73
25	15	5.9	0.4	2.9	193	1.1	73
26	15	6.5	0.4	1.8	120	0.6	43
27	10	12.4	1.2	1.3	130	3.5	350
28	4	8.8	2.2	1.7	425	10.3	2575
29	3.3	8.5	2.6	2.2	667	14.0	4242
30	2	5.6	2.8	0.6	300	4.6	2300
31	2	5.7	2.8	2.5	1250	3.9	1950
32	2	9.7	4.8	3.7	1850	13.4	6700
33	2	4.7	2.3	1.8	900	13.7	6850
Mean of 12 samples		8.3	1.8	2.2	529	6.8	2184
" " 5-15 U samples		8.9	0.6	2.5	165	3.7	250
" " 4-2 U "		6.4	3.2	2.2	1075	8.4	3875
34*		189.4†		23.0†		223.2†	
35†		151.8†		29.8†		198.1†	

* Unfractionated crude concentrate obtained by hot water (pH 5.0—85°C) extraction of beef liver.

† Unfractionated crude concentrate obtained by hot water (pH 5.0—85°C) extraction of pork liver (90% pork—10% beef).

‡ Amount present in crude concentrate obtained from 100 g of liver.

been determined by the neurospora method of Horowitz and Beadle.⁴

Discussion. As previously reported¹ for riboflavin, niacin, pantothenic acid, and folic acid (*L. casei* factor), and as can be seen from Table I, the various extracts show considerable variation in their content of pyridoxine, choline, and *l*-methionine. Before discussing the parenteral liver extracts it might be well to see how much pyridoxine, choline, and *l*-methionine is extracted from whole liver by the usual hot (85°C.), acid-water (pH 5.0) treatment. The values for unfractionated beef liver concentrate (34) and unfractionated pork liver concentrate (35) are shown at the bottom of Table I. Using the average for both beef and pork, we obtain 189 μ g of pyridoxine, 223 mg of choline and 23 mg *l*-methionine from 100 g of whole liver. Using these values as a "yardstick," it becomes evident that the addition of ethyl alcohol to make 65-70%, by volume, together with the other procedures used in the prepara-

tion of suitable parenteral extracts, removes a large part of each of the several B factors. Without definite knowledge as to the amount of liver used in the preparation of various extracts, it is not possible to estimate the percentage of the various factors present in the finished preparations. The data presented in Table I shows the amount of each of the 3 substances per cc and per U.S.P. injectable unit. In the nonspecific uses of liver extract very little attention is paid to the antianemia potency; therefore, the best evaluation of the different samples and types of liver extract is the comparison of the amount of each factor present in 1 cc.

Using the average value of 5 concentrated extracts, the pyridoxine present per cc is approximately 5% of that originally present in the crude concentrates, 34 and 35; the choline is less than 2%; the *l*-methionine is about 9%. Using the average value of 4 crude extracts, the pyridoxine per cc is about 4%; the choline 4%; and the *l*-methionine 8%. Actually on a cc basis, there is no significant difference between the pyridoxine and *l*-meth-

⁴ Horowitz, N. H., and Beadle, G. N., *J. Biol. Chem.*, 1943, **150**, 325.

ionine content of the refined, concentrated extracts and the dilute, crude preparations. There is definitely more choline in the crude extracts.

Since we are dealing with the extraction of preformed substances stored in the liver and since nutrition of the animal affects the storage of such substances, it is obvious that the data presented are relative rather than absolute.

Summary. From further quantitative studies of U.S.P. refined, concentrated and from dilute, crude liver extracts, it is evident, using average values per cc, that the amounts of pyridoxine and *l*-methionine are approximately the same in the two types of extract. The crude extracts contain more choline than the refined preparations.

The writer is indebted to Alice Craig and Maurice Avery for the analytical data presented.

16540

A Differential Response of the Rodent Adrenal Gland to Acute Starvation.

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It has been shown that the adrenal gland of the female guinea pig progressively enlarges during starvation and that this hypertrophy requires the presence of an intact hypophysis and cannot be appreciably overcome, in the starved animal, with cortical hormone overdosage.¹ The experiments of Cameron and Carmichael² have indicated that enlargement of the adrenals in the rat occurs only in the advanced stages of acute starvation, and that this increase in size is not a true physiological growth but is rather a pathologic change involving increased water content. The present report describes preliminary experiments on rats and guinea pigs which demonstrate differences in adrenal response to acute inanition.

Materials and Methods: Female guinea pigs (290-320 g) and adult rats of both sexes (150-250 g) were subjected to acute starvation over a period of 6-11 days by complete food deprivation (water allowed freely). During starvation some animals were given daily subcutaneous injections of 5-18 mg of desoxycorticosterone acetate (DOCA) in saline or

in sesame oil.* Starved controls received injections of saline or sesame oil alone or remained untreated. Corresponding groups of animals received similar experimental treatment and the complete normal laboratory ration. All animals were exsanguinated 24-36 hours after the last injection, or sooner, if moribund. The adrenals were removed, trimmed, weighed in the fresh state, and dried for 24-36 hours at 100-105° C to constant weight for solid and water content.

Results. The data (tables) indicate that the morphologic response of the adrenals to acute starvation is different in the guinea pig and the rat. In the former, the adrenal gland shows an absolute increase in size and in solid content with advanced starvation. The water content of these hypertrophied glands increases proportionately, and the percentages of solids and water are not appreciably changed from the normal. In contrast, the adrenals of acutely starved rats often fail to enlarge or may diminish in size. Likewise, the solid content of the gland does not increase but either remains unchanged or may

¹ D'Angelo, S. A., Gordon, A. S., and Charipper, H. A., *Endocrinology*, 1948, **42**, 399.

² Cameron, A. T., and Carmichael, J., *Canad. Research*, 1946, **24**, 37.

* Dr. B. J. Brent, Roche Organon Inc., and Dr. Erwin Schwenk, Schering Corporation, kindly supplied the hormone.

TABLE I.
The Effect of Desoxycorticosterone Acetate on the Adrenals of the Normally Fed and Acutely Starved Rat.

Treatment	Sex	No.	Mean body wt, g			Mean adrenal wt, mg	Mean water content, %	Mean solid content, mg	Mean adrenal solids, mg/100 g body wt	
			Initial	Final	% loss				Initial	Final†
Normal fed, saline 1 cc 9 days	♀	9	160 ± 20*	175 ± 19		42.3 ± 5.5	65.5 ± 3.6	15.3 ± 0.9	6.57 ± 0.6	8.25 ± 0.7
Starved, saline 0.5-1.5 cc daily, 8-9 days	♀	9	178 ± 14	101 ± 13	43.2 ± 5.1	42.8 ± 5.0	73.8 ± 1.2	12.4 ± 0.8		12.05 ± 2.2
Normal fed, DOCA, 10 mg daily, 8 inj.	♀	6	166 ± 19	178 ± 20		26.3 ± 5.2	61.6 ± 0.9	11.9 ± 0.1		5.90 ± 0.6
Starved, DOCA, 15 mg daily, 8-9 inj.	♀	6	168 ± 19	102 ± 12	39.2 ± 2.1	30.1 ± 9.0	64.6 ± 9.2	9.6 ± 2.3	5.25 ± 1.6	8.57 ± 2.6
Starved, DOCA, 5 mg daily, 8 inj.	♀	3	183 ± 21	110 ± 20	40.0 ± 5.1	39.7 ± 6.0	68.8 ± 5.8	11.8 ± 0.7	7.12 ± 1.0	12.75 ± 2.8
Normal fed, saline 1 cc 9 days	♂	6	192 ± 21	207 ± 19		26.1 ± 3.0	65.6 ± 2.2	9.3 ± 1.2		4.40 ± 0.5
Starved, saline 0.5-1.5 cc daily, 8-9 days	♂	6	220 ± 15	134 ± 16	39.1 ± 5.2	26.9 ± 6.5	69.7 ± 6.5	8.1 ± 0.9	3.69 ± 0.2	6.18 ± 0.8
Normal fed, DOCA, 10 mg daily, 8 inj.	♂	3	197 ± 16	214 ± 17		17.0 ± 0.7	68.6 ± 0.9	5.5 ± 0.1		2.50 ± 0.2
Starved, DOCA, 15 mg daily, 8-9 inj.	♂	4	206 ± 15	133 ± 12	35.4 ± 4.1	13.3 ± 1.8	67.0 ± 5.0	4.1 ± 1.1	1.90 ± 0.4	2.94 ± 0.5
Starved, DOCA, 5 mg daily, 8 inj.	♂	3	209 ± 24	125 ± 20	40.1 ± 2.8	24.9 ± 4.0	66.5 ± 8.0	7.3 ± 3.3	3.10 ± 0.6	5.04 ± 1.9

* Refers to the mean ± average deviation.

† In all fed animals, the ratio is calculated to final body wt only.

TABLE II.
Comparison of Water and Solid Content of Adrenal Glands in the Normal and Acutely Starved Female Guinea Pig.

Treatment	No. of animals	Days starved	Mean body wt, g			Mean adrenal wt, mg	Mean water content, %	Mean solid content, mg	Mean adrenal solids, mg/100 g body wt	
			Initial	Final	% loss				Initial	Final
Fed, untreated	6	0	311 ± 7*			118 ± 13	66.4 ± 1.2	39.3 ± 4.3		12.6 ± 1.3
Starved	5	6-8	310 ± 15	191 ± 19	38.4 ± 2.4	187 ± 32	68.6 ± 3.8	57.2 ± 5.9	18.5 ± 1.8	30.3 ± 4.2
Fed, saline 1.5 cc daily, 10 days	7	—	304 ± 9	348 ± 22		157 ± 22	68.4 ± 1.6	49.6 ± 4.0		14.2 ± 0.7
Fed, DOCA, 15 mg in saline, daily, 10 days	3	—	307 ± 8	339 ± 12		154 ± 10	67.0 ± 0.3	49.5 ± 3.7		15.0 ± 1.1
Starved, sesame oil, 0.4 cc daily	6	7-9	301 ± 8	193 ± 10	35.9 ± 3.8	181 ± 24	67.4 ± 2.3	50.2 ± 2.0	16.3 ± 0.3	28.4 ± 1.5
Starved, DOCA, 10-18 mg in sesame oil	6	6-7	306 ± 8	201 ± 14	34.3 ± 2.6	234 ± 45	70.0†	63.0	20.5	31.3

* Mean ± average deviation.

† Determined in 2 animals.

‡ Single determination.

be reduced (females).† Occasionally the adrenals of rats in advanced stages of acute starvation do show enlargement. This is especially the case in moribund or dead animals. These glands are usually discolored (grey, maroon) and analysis indicates that the increased size is due largely to increased water content. The adrenals of moribund guinea pigs are heavier than glands of starved pigs with similar body weight loss. This "death hypertrophy" involves an increase in both water and solids with no significant alteration in the normal percentages of these constituents.

The administration of DOCA (10-15 mg daily) decreases the size of the adrenals in acutely starved and fed rats. In either instance, a significant diminution in solid content occurs. The color of these adrenals is usually pale yellow even in terminal stages of starvation. DOCA in the guinea pig is without effect on adrenal size in the fed animal, and fails to prevent adrenal hypertrophy in the

† The ratio of adrenal solid content to final body weight, however, is increased over the pre-starvation ratio. In this sense, the starved rat adrenal displays relative rather than absolute hypertrophy.

starved guinea pig. Although only several analyses have been done in this series of animals, it appears that the solid and water content of the adrenals are not affected by DOCA injection.

A detailed histologic study is now being made of the adrenal and pituitary glands of the animals described in this report. These observations together with a more extensive discussion of the problem and consideration of the literature will be published at a future date.

Summary. The response of the adrenal gland to comparable degrees of acute starvation differs markedly in the rat and guinea pig. The guinea pig adrenal displays absolute hypertrophy with significantly increased solid content. In the rat, adrenal enlargement is a variable response and, when it occurs, is characterized by discoloration and percentage increase in water content. The solid content is unchanged or reduced. DOCA in high doses decreases the size and the solid content of the rat adrenal in the fed or acutely starved animal of either sex. DOCA in the guinea pig fails to affect adrenal size in the fed animal, and is ineffective in preventing the adrenal hypertrophy of starvation.

16541

Effect of Desoxycorticosterone upon Hypophyseal Corticotrophin Production.

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The atrophy of the adrenal cortex produced by chronic administration of cortical extract was shown to be counteracted by the simultaneous administration of adrenocorticotrophic hormone (ACTH);¹ presumably the corticoids merely inhibit hypophyseal corticotrophin production without rendering

the cortex insensitive to the trophic hormone. A similar involution of the adrenal cortex in both the rat and the mouse was obtained with desoxycorticosterone acetate (DCA).² Sayers and Sayers³ showed that the depletion of the adrenal ascorbic acid which follows the exposure of rats to stress can be prevented by the administration of

* Aided by a fellowship from the Dazian Foundation for Medical Research.

¹ Ingle, D. J., Higgins, G. M., Kendall, E. C., *Anat. Rec.*, 1938, **71**, 363.

² Selye, H., *J. Am. Med. Assn.*, 1940, **115**, 2246.

³ Sayers, G., and Sayers, M. A., *Endocrinology*, 1947, **40**, 265.

TABLE I.

Exp.	Group	No. of rats	Treatment	Adrenal ascorbic acid	
				mg/100 g fresh tissue (mean \pm stand. error)	% decrease from normal control
1	I	9	DCA + cold	320 \pm 12.0	12
	II	10	Cold	320 \pm 13.6	12
	III	10	Normal control	362 \pm 12.3	—
2	I	10	DCA + cold	259 \pm 15.0	18
	II	10	Normal control	317 \pm 14.4	—

DCA. They concluded that the production of ACTH by the pituitary is inversely proportional to the concentration of corticoids in the body fluids and corresponds to the cortical-steroid requirements of the peripheral tissues.

In the present communication we endeavor to show that this mechanism of pituitary regulation is not effective in the presence of very high concentrations of DCA in the body fluids.

The anesthetic effect of this steroid^{4,5}—which is only obtained with excessive doses—served to establish that the amount administered was greatly in excess of the animals' requirements even under stress. The adrenal response to the treatment was estimated by determining the ascorbic acid concentration in the adrenals of all animals at the end of each experiment.³

Thirty male albino rats (80-100 g), fasted for 24 hours, were divided into 3 groups of 10 rats each. The first group received intraperitoneally 6 mg of DCA dissolved in warm corn oil and repeated doses of 2 mg were similarly administered to each animal to maintain it in a state of anesthesia during the 6 to 7 hours of the experiment. These rats were intermittently exposed to cold ($10 \pm 1^\circ\text{C}$) for one hour at a time and between exposures they were allowed to recuperate at room temperature for about half an hour. During the rest periods all animals about to awaken received an additional dose of 2 mg of DCA and those rats which were profoundly narcotized were allowed longer rest periods.

Most of the animals were exposed for a total of 4 hours and none for less than 3; the total dosage of DCA administered ranged from 12 to 14 mg per animal. Group 2 received exactly the same cold treatment as group 1 but no DCA was administered to it. Group 3 received no treatment and thus served as an absolute control. At the end of the experimental period all animals were sacrificed and the adrenal ascorbic acid determined by Carruthers's method.⁶ The results are summarized in Table I as Experiment 1.

Experiment 2 represents the results obtained with a similar experimental arrangement in which the cold-exposure period was increased to $4\frac{1}{2}$ hours and the DCA dosage to 16 mg per animal.

Both in Experiment 1 ($P < 0.05$) and in Experiment 2 ($P < 0.02$) the difference in adrenal ascorbic acid between the DCA-treated, cold-exposed group and the normal controls is significant.

The results reported above show that in the presence of a high tissue concentration of DCA, the pituitary of animals exposed to stress still elaborates sufficient ACTH to induce a significant decrease in adrenal ascorbic acid. In Experiment 1 the exposure to cold alone (Group II) brought about a decrease of the same order as in the animals (Group 1) which simultaneously with the cold treatment received the DCA overdosage. These findings are incompatible with the assumption that pituitary adrenocorticotrophin discharge is regulated solely by the corticoid concentration in the body fluids. Our observations point to the possibility that in the presence of ex-

⁴ Selye, H., *Proc. Soc. Exp. Biol. and Med.*, 1941, **46**, 116.

⁵ Selye, H., *Anesth. a. Analg.*, 1942, **21**, 42.

⁶ Carruthers, C., *Ind. Eng. Chem. Anal. Ed.*, 1942, **14**, 826.

cessively high corticoid concentrations—as at times of very acute stress—the regulation of the corticotrophin discharge may become relatively independent of circulating cortical hormone(s).

Summary. In the rat large doses of desoxycorticosterone acetate do not prevent the decrease in the concentration of adrenal ascorbic acid which accompanies exposure to cold. It

is suggested that during severe stress, corticotrophin discharge is conditioned by factors other than mere lack of corticoids.

This experiment was subsidized by a grant from the Commonwealth Fund.

The authors are also grateful to the Schering Corp. of Bloomfield, N. J., for supplying the desoxycorticosterone acetate.

16542

Adenochrome-Like Pigment of the *Polyzoa bugula neritina* (L).

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Bugula neritina (L), a *Polyzoa* (Bryozoa) of the order *Cheilostomata*, presents a dark purple pigment not commonly found in other animals of the same order.¹ As far as we know no study of the pigments of *Polyzoa* has yet been reported.^{2,3} Our specimens were collected in the bay of Rio de Janeiro near the Hydrobiological Station of the Institute.

The pigment of *Bugula* appears morphologically in the granular corpuscles of the distal part of the *zooecium* (Fig. 1) and is easily extracted with distilled water. The extract is wine-red in color and shows a Tyndall effect and a pH of 6.8.

The physical and chemical properties investigated showed that the pigment closely resembles the adenochrome isolated by Fox and Updegraff from the branchial hearts of the *Octopus*.⁴ The absorption spectrum, however, exhibits a different maximum (545 m μ as compared with 505 m μ for the adenochrome).

Fox and Updegraff suggested that adenochrome is probably a waste product, as shown by its localization in the excretory tissues of the *Octopus* and the apparent storage

of most of its nitrogen in the amide state, releasable as free ammonia in the presence of KOH.⁴ In *Bugula* the pigment is accumulated in the distal part of the *zooecium* and around the *brown body* which is known to be an excretory waste mass. However, until now we have been unable to establish any relation between the pigment and the excretory function of *Bugula*. Other water-soluble pigments behaving as indicators of pH have been reported by Crozier in two sponges and a nudibranch but unfortunately no detailed chemical and spectroscopical data were published.⁵

Isolation and properties of the pigment. Animals from a colony (about 30 g) were washed with filtered sea water to free them of associated organisms, then weighed and extracted with boiling distilled water for 5 minutes. The residue was reextracted with boiling water until the solution was only slightly yellow. The extract was filtered and washed with petroleum ether (b.p.40-60°). The aqueous phase was made alkaline (pH 11.0) with KOH and the flocculent purple pigment was separated by centrifugation. The precipitate was dissolved in 10% acetic acid, then diluted with a few ml of

¹ Marcus, E., *Bol. Fac. Fil., São Paulo*, 1937, **1**, 67.

² Lederer, E., *Biol. Rev.*, 1940, **15**, 273.

³ Fox, D. L., *Ann. Rev. Bioch.*, 1947, **16**, 443.

⁴ Fox, D. L., and Updegraff, D. M., *Arch. Biochem.*, 1942-43, **1**, 339.

⁵ Crozier, W. J., *J. Biol. Chem.*, 1918, **35**, 455.



FIG. 1.

Left: Fresh preparation showing the distal distribution of the pigment in the zoecium (arrows). $\times 50$. Right: Microphotography of the pigmented corpuscles. $\times 180$.

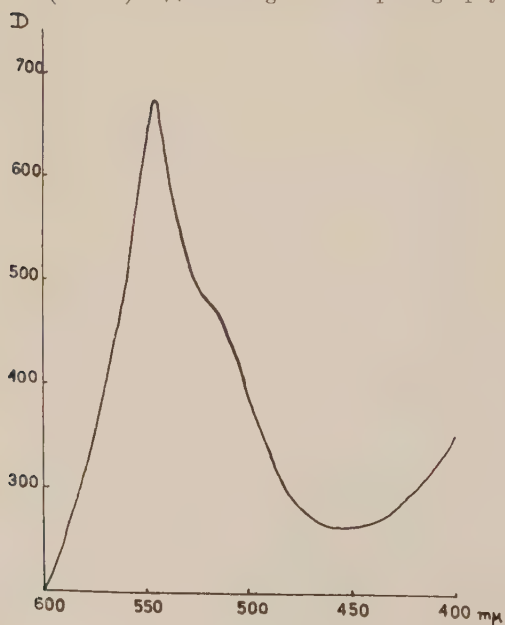


FIG. 2.

Absorption spectrum of the pigment in water at pH 6.8. Ordinate: optical density. Abscissa: wavelength in $m\mu$.

water and reprecipitated with 4 N KOH. After centrifugation the precipitate was washed 3 times with 95% ethanol, once with ether and finally dried at 37°C . From 30 g of washed animals 21 mg of dry pigment were isolated. The powder had no melting point. Large variations in the pigment content were observed among individual colonies, the darker being richer than those presenting a pink color.

The absorption spectrum was determined with a Beckman spectrophotometer and the absorption curve showed a sharp maximum at $545 m\mu$, the concentration being 0.6 g/1000 and the pH 6.8. Fig. 2 shows a typical curve.

Since the color and the stability of the solutions of the pigment depend on the pH, titrations with alkali and acid were made to determine the behaviour of the solution at different H-ion concentrations. A water solution containing 4 mg of the pigment in 10 ml of solution was titrated with 0.2 N NaOH or

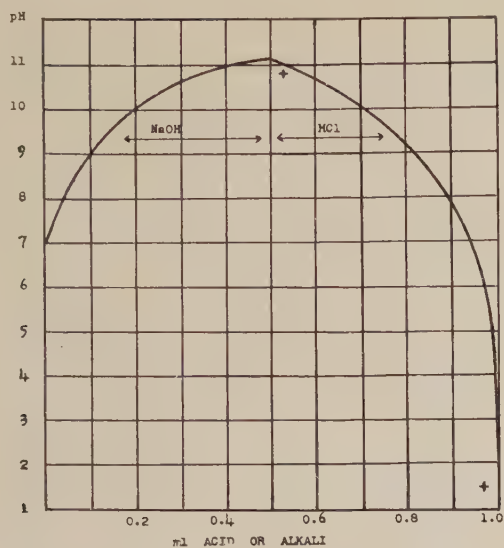


FIG. 3.

Titration curve of the pigment dissolved in water. Ordinate: pH units. Abscissa: ml of standard alkali or acid (0.2 N).

HCl solutions and the pH determined at intervals with a Macbeth pH-meter equipped with glass electrodes. At pH 11.1 all the pigment was precipitated and back titration to pH 9.2 with standard acid redissolved it; it reprecipitated at pH 2.0. A titration curve is shown in Fig. 3 and the precipitation zones are indicated with a cross. Inspection of this curve reveals the marked buffering capacity of the dissolved pigment. The changes of color with pH are recorded in Table I.

The pigment is soluble in water, very solu-

TABLE I.

pH	Color
2.4	blue-purple
3.0	purple
5.0	red-wine
6.8	red-wine
8.0	red
9.5	purple
11.0	purple-blue

ble in alkaline solutions, but insoluble in the common organic solvents. Addition of acetone to the aqueous extract (10 volumes of acetone to 1 volume of the extract) precipitates the pigment. The dried precipitate is easily dissolved in alkali or acid. When water solutions of the pigment are treated with concentrated KOH or NaOH small bubbles develop which react with Nessler reagent. Fox and Updegraff reported a similar reaction with adenochrome.

The pigment of *Bugula* is adsorbed on MgO, Ca(OH)² and CaCO₃. It was possible to liberate it from the CaCO₃ with 10% acetic acid solution. Strong adsorption of the pigment was also obtained on Brockmann's aluminum hydroxide, but all of the solvents tried failed to extract it from the adsorbate. Zinc dust in KOH and sodium hydrosulfite produced rapid decolorization. KMnO₄ and H₂O₂ also discharged the color, which partially returned after the addition of sodium hydrosulfite. Biuret and xanthoproteic reactions were negative. The nihydrin test was positive. The murexide test for purines and the Salkowski test for indole were negative. Boiling water failed to precipitate the pigment even when acidified with acetic acid. Reduced sulfur was absent and the Liebermann test for phenol was negative.

Summary. A water soluble pigment was isolated from the *Polyzoa Bugula neritina* (L) and its chemical and physical properties were determined. A close resemblance was verified between this pigment and the adenochrome obtained from the branchial hearts of the *Octopus* by Fox and Updegraff.

We wish to express our thanks to Dr. W. Cruz of the Instituto Oswaldo Cruz for some spectrophotometric determinations and to Prof. P. Drach, Roseoff Marine Laboratory, for his interest during the progress of this work.

Use of Sodium Azide to Control Bacterial Destruction of Rh Agglutinating Antibodies.

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(Introduced by Maurice Landy.)

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Numerous authors have stated that errors in blood grouping, particularly false positive reactions, may result from the use of contaminated blood grouping serum.^{1,2,3,4} With the increasing use of anti-Rh serums in routine diagnostic tests as well as in numerous phases of investigative work it becomes increasingly important to have a satisfactory preservative for such serums. Waller⁵ reported that merthiolate inhibited anti-Rh agglutinins, but was without effect on the other hemagglutinins tested. Diamond and Abelson,⁶ however, noted that exposure to merthiolate "while inactivating anti-Rh agglutinins, appears not to affect" Rh "blocking" antibodies. Cameron and Diamond⁷ found that anti-Rh serum containing 0.1% carboxylmethoxylamine hemi-hydrochloride ("compound I") showed some loss of both titer and avidity after storage at room temperature, but it is not clear whether the slight loss of potency was due to the storage temperature or to the presence of "compound I". No additional reports on the use of preservatives specifically in anti-Rh serums have appeared, although Favour⁸ suggests that

"compound I" can be used "in the preservation of blood-typing serum, . . . and other preparations with a high protein content."

The successful use of sodium azide (NaN_3) in preserving liquid complement,⁹ and its broad spectrum of bacteriostatic and fungistatic activity^{10,11,12} suggested the possibility of its use in the preservation of anti-Rh serums, and is the subject of this investigation.

Materials and Methods. For the experiments reported here a high-titered human serum was selected containing anti-Rh₀ (anti-D) saline agglutinins. These antibodies are known to be more labile than are the "blocking" (univalent) type of Rh antibodies and thus should provide a more sensitive measure of any deleterious action of a preservative. The serum was diluted to a titer† of 128, using as the diluent pooled normal serum containing neither Rh nor other irregular isohemagglutinins. The serum was sterilized by filtration and divided into three portions. To two of these NaN_3 was added to a concentration of 0.1% and 0.05% respectively. The third portion was retained as the untreated control. Each of these three portions was then further subdivided; half was kept sterile and the other half was seeded with a mixed suspension of bacteria composed of five strains originally isolated as common

* Captain M. C., A. U. S.

1 Davidsohn, I., and Toharsky, B., *J. Infect. Dis.*, 1940, **65**, 25.

2 Davidsohn, I., and Toharsky, B., *J. Immunol.*, 1942, **43**, 213.

3 Grove, E. F., and Crum, M. J., *J. Lab. & Clin. Med.*, 1930, **16**, 259.

4 McGraw, J. J., Vaubel, E. K., and Elliott, J., *Surg. Clin. N. America*, 1945, **25**, 1042.

5 Waller, R. K., *Am. J. Clin. Path.*, 1944, **14**, 116.

6 Diamond, L. K., and Abelson, N., *J. Clin. Inv.*, 1945, **24**, 122.

7 Cameron, J. W., and Diamond, L. K., *J. Clin. Inv.*, 1945, **24**, 793.

8 Favour, C. B., *J. Bact.*, 1948, **55**, 1.

9 Richardson, G. M., *Lancet*, 1941, **241**, 696.

10 Lichstein, H. C., *J. Bact.*, 1941, **42**, 293.

11 Lichstein, H. C., and Soule, M. H., *J. Bact.*, 1944, **47**, 221.

12 Herrick, J. A., and Kempf, J. E., *ibid.*, 1944, **48**, 331.

† Reciprocal of greatest dilution giving macroscopic agglutination of test cells.

TABLE I.

Agglutinin Titers of Sterile Anti-Rh₀ Serum (Duplicate Samples) Preserved with Graded Quantities of NaN₃ and Stored at 37°C. Test Cell Suspensions and Serum Dilutions Made in 0.85% NaCl.

CONCENTRATION OF NaN ₃	WEEKS OF STORAGE	SERUM DILUTION							
		2	4	8	16	32	64	128	256
0.1%	0	++++	++++	++++	++++	++++	+++	+	-
	8	++++	++++	++++	+++	++	-		
		++++	++++	++++	+++	++	-		
	10	++++	++++	+++	+++	+	-		
		++++	++++	+++	++	-			
0.05%	0	++++	++++	++++	++++	++++	+++	+	-
	8	++++	++++	+++	++	+	-	-	
		++++	++++	++++	+++	++	-	-	
	10	+++	+++	+++	+++	++	-	-	
		++++	+++	+++	+++	++	-	-	
NONE (CONTROL)	0	++++	++++	++++	++++	++++	+++	++	-
	8	++++	++++	+++	++	+	-		
		++++	++++	+++	++	+	-		
	10	+++	+++	+++	++	-			
		++++	+++	+++	++	+	-		
	14	+++	+++	-					
		+++	++	-					
		+++	+++	-					
		+++	+++	-					
		+++	+++	-					

Symbols: ++++ Single large clump.
 +++ Breaks into several large clumps.
 ++ Breaks into numerous small clumps.
 + Many fine macroscopic clumps.
 - Negative.

laboratory contaminants.† The suspensions were standardized photometrically to contain approximately 100 million organisms per cc and a sufficient quantity was added so that the serums contained approximately one million organisms per cc. Each of the six samples was tested for sterility by inoculation of tubes of veal infusion broth, titrated for serological activity, and dispensed aseptically into 5 cc vaccine bottles fitted with rubber diaphragm sleeve stoppers. Duplicate samples of each series of treated and untreated (NaN₃), sterile and contaminated serums were then stored at 37°C. At periodic intervals all samples were titrated against a 2% saline suspension of pooled Group O, Rh₀ positive (D positive) cells, and tested for sterility. Results of these serological titrations are pre-

sented in Tables I and II.

Results. It was found that NaN₃ in the concentrations tested was bacteriostatic, though not bactericidal for the test organisms. Viable organisms were found at all test periods. The only strain which was recovered consistently from the NaN₃ treated samples was the streptococcus. Only Gram negative bacilli were recovered from the untreated contaminated control specimens although microscopic examination of direct smears revealed the presence of Gram negative bacilli, Gram positive bacilli and streptococci.

Periodic titrations revealed a gradual decrease in potency (loss of titer) in both the NaN₃ treated and untreated sterile samples. This decrease in potency in the two series was approximately parallel, indicating that NaN₃ had no observable influence on the thermal stability of the antibody being studied.

In the contaminated series, however, a marked difference in potency was noted after 3 weeks storage: the samples containing NaN₃ showed a slight loss of titer, comparable

† The cultures used were *Bacillus cereus*, *Aerobacter cloacae*, *Streptococcus liquefaciens*, *Pseudomonas ovalis*, and *Staphylococcus epidermidis*. These were obtained from the National Institute of Health through the courtesy of Dr. Margaret Pittman.

TABLE II.

Agglutinin Titers of Contaminated Anti-Rh₀ Serum (Duplicate Samples) Preserved with Graded Quantities of NaN₃ and Stored at 37°C. Test Cell Suspensions and Serum Dilutions Made in 0.85% NaCl.

CONCENTRATION OF NaN ₃	WEEKS OF STORAGE	SERUM DILUTION							
		2	4	8	16	32	64	128	256
0.1%	0	++++	++++	++++	++++	++++	+++	++	—
	3	++++	++++	+++	++	++	+	—	
		++++	++++	+++	++	+	+	—	
	8	++++	++++	+++	++	+	—		
		++++	++++	+++	++	+	—		
	10	++++	++++	+++	++	+	—		
0.05%	0	++++	+++	++	—				
		++++	++	+	—				
	3	++++	++++	+++	++	++	+	—	
		++++	++++	+++	++	++	+	—	
	8	++++	++++	+++	++	—			
		++++	+++	+++	+	+	—		
NONE (CONTROL)	0	++++	++++	+++	++	+			
		++++	+++	++	+	—			
	3	+++	+	—					
		++	—	—					
	8	—	—	—					
		—	—	—					

SYMBOLS USED HAVE THE SAME MEANING AS GIVEN IN THE LEGEND TO TABLE 1.

to that found in the sterile samples, whereas the contaminated samples stored without NaN₃ showed a more striking and rapid loss of titer.

Complete inactivation of the Rh antibody in the untreated contaminated samples was observed after 10 weeks storage whereas the sterile samples and contaminated samples containing NaN₃ were still very active, although showing some loss of titer due to the storage temperature.

It is apparent from these experiments that bacterial contamination of anti-Rh serum can, under some circumstances, result in complete inactivation of Rh antibodies. NaN₃ in concentrations of 0.05% and 0.10% inhibited this inactivation. It is doubtful that the inactivation of hemagglutinins by bacteria observed here is specific for Rh antibodies,[§] rather it is probably due to gradual destruction of the immune globulins of the serum, possibly through proteolysis.

Despite the toxicity^{||} which may limit its

§ In preliminary experiments the same effect of bacterial contamination has been demonstrated on "blocking" Rh antibodies. Similar studies are being carried out with the alpha and beta isoagglutinins. The identity of the strain or strains of organisms which caused deterioration of the antibodies has not been established.

|| Although NaN₃ is highly toxic,^{13,14,15} the small amount present (1 to 5 mg) in the volume of anti-Rh serum usually dispensed would scarcely represent any danger to the technician. Cf. Graham *et al.*¹⁶ who recommends further investigation of this chemical as a therapeutic agent.

¹³ Kempf, J. E., and Nungester, W. J., *Science*, 1944, **100**, 411.

¹⁴ Fairhall, L. T., *et al.*, *Pub. Health Reports*, 1943, **58**, 607.

¹⁵ Smith, L., and Wolf, C. G. D., *J. Med. Res.*, 1904, **7**, 451, cited by Richardson.⁹

¹⁶ Graham, J. D. P., Rogan, J. M., and Robertson, D. G., *J. Indust. Hyg. & Toxicology*, 1948, **30**, 98.

usefulness, NaN_3 could be of value in the preservation of anti-Rh serums. It possesses certain advantages over "compound I" in that it is active against fungi, and is effective in heavily contaminated materials. Like "compound I" the effectiveness of NaN_3 is not impaired by high concentrations of protein. In experiments now in progress it is being found that the bacteriostatic effectiveness of NaN_3 is not impaired in anti-Rh serums fortified with 20% bovine albumin. Through their action as specific metabolic inhibitors, compounds such as NaN_3 and "compound I"

may find wider use in the preservation of diagnostic biologicals.

Summary. The effectiveness of NaN_3 in concentrations of 0.1% and 0.05% as a preservative for anti-Rh agglutinating serums has been investigated. It was found that in these concentrations the agent was markedly bacteriostatic, but had no apparent effect on the titer or avidity of the antiserum. Anti-Rh serum deliberately contaminated with common bacteria but not preserved with NaN_3 rapidly lost potency when stored at 37°C.

16544

Effects of Ovarian Hormones on Certain Cytoplasmic Reactions in the Vaginal Epithelium of the Mouse.*†

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Since the classical work of Allen and Doisy¹ many investigators have studied the effects of the various gonadal hormones on the growth and differentiation of the vaginal epithelium. With the exception of the numerous studies on glycogen in the monkey and human and mucin in the rodent, little work has been directed toward the alterations in intracellular constituents. Recently Jeener² stated in a brief report that estrogen evoked a marked increase in both alkaline phosphatase and cytoplasmic ribonucleic acid in the vaginal epithelium of the mouse, but few cytological details were given.

* This investigation has been aided by a grant from the Jane Coffin Childs Memorial Fund for Medical Research and by a grant from the Rockefeller Foundation administered by Dr. Philip E. Smith.

† The estradiol benzoate used in this work was supplied through the courtesy of Schering Corporation, the progesterone by the Warren-Teed Products Company.

¹ Allen, E., and Doisy, E. A., *J.A.M.A.*, 1923, **81**, 819.

² Jeener, R., *Nature*, 1947, **159**, 578.

In view of this paucity of information, the present study was designed to examine the effects of estrogen and progesterone on the amount and distribution of cytoplasmic ribonucleic acid, alkaline phosphatase and mucin in the vaginal epithelium of the mouse.

Methods. Twelve young adult mice of the Swiss albino strain were bilaterally ovariectomized. A control group of 3 animals received no further treatment and was sacrificed 3 to 4 weeks after operation. At this time the remaining mice were divided into 3 equal groups. One group received 1.0 μg of estradiol benzoate; the second, 1.0 mg of progesterone, and the third received simultaneously 1.0 μg of estradiol benzoate and 1.0 mg of progesterone daily for 3 to 5 days. Crystalline hormones were used. The daily dose was dissolved in 0.05 to 0.1 cc of sesame oil and was injected subcutaneously. The animals were sacrificed on the day following the last injection and were autopsied immediately. The vaginas from both the control and experimental groups were fixed in chilled 80% ethyl alcohol for at least 24

hours and were subsequently dehydrated and embedded in paraffin. Tissue sections were cut 7 microns in thickness.

Alkaline phosphatase activity was determined by incubating sections in a medium containing 1% sodium glycerophosphate at pH 9 according to the technic of Gomori.³ The original formula was altered slightly by the addition of 0.01 M magnesium sulfate which is an alkaline phosphatase activator. The tissue was incubated at 37°C for 1½ hours. Subsequent treatment results in the deposition of fine black particles of cobalt sulfide at the sites of enzyme activity. The sections were counterstained with eosin and were mounted in clarite dissolved in white gasoline.

Ribonucleic acid was demonstrated by staining sections in 0.01% toluidine blue in a citrate-phosphate buffer at pH 5 for one hour at 37°C. In order to ascertain the amount of staining due specifically to ribonucleic acid, occasional parallel sections were first incubated in a solution of crystalline ribonuclease (1 mg/cc buffered at pH 6.8 for 1 hour at 56°C) before staining in the above manner. The sections were rinsed in distilled water, dehydrated and mounted in clarite.

For the identification of mucin, sections were first incubated with saliva for 5 minutes to remove any glycogen which might be present. The sections were then oxidized with chromic acid and treated with leucofuchsin according to the Bauer-Feulgen technique.⁴ Mucin is stained a bright red by recolorized basic fuchsin. The nuclei were counterstained lightly with Weigert's iron hematoxylin and the sections mounted in the usual manner.

Results. As stated in the introductory remarks, numerous authors have dealt with the cytological changes in the vaginal epithelium due to the action of gonadal hormones. The writers have found the following classification of morphological patterns based on the rat (Freud⁵) to be most generally useful:

³ Gomori, G., *J. Cell. & Comp. Physiol.*, 1941, **17**, 71.

⁴ Bauer, *Ztschr. mikr. anat. Forsch.*, 1933, **33**, 143.

⁵ Freud, J., *Act. Brev. Neerland.*, 1938, **8**, 127.

Type I. Epithelium 2 to 3 cells thick. The long axes of the cells in the most superficial layer tend to be perpendicular to the surface of the epithelium. Found in infantile, lactating and castrate animals.

Type II. Multilayered stratified epithelium without mucification or cornification.

Found in diestrus and proestrus and in the early stages of estrogen treatment in the castrate.

Type III. Multilayered stratified epithelium with mucification of all but the most basal cells.

Found in pregnancy and pseudopregnancy and in the castrate treated with estrogen and progesterone concurrently.

Type IV. Multilayered stratified epithelium with cornification of the more superficial layers. The deeper cells may be differentiated into a stratum germinativum and stratum granulosum.

Found in estrus and in the estrogen treated castrate.

The only variation from the descriptions of Freud which occurred in our series was in the estrogen-progesterone treated group and will be discussed below.

The present histochemical studies reveal that there is no alkaline phosphatase or mucin present in the vaginal epithelium of the ovariectomized mouse. Fine granules containing small amounts of ribonucleic acid are irregularly scattered throughout the cytoplasm of the epithelial cells. There is no change in the status of any of these cellular constituents after treatment with progesterone alone.

After treatment with estrogen, however, there is a marked increase both in alkaline phosphatase and ribonucleic acid. Heavy deposits of cobalt sulfide, indicating strong enzyme activity, are present in the nuclei and throughout the cytoplasm of the cells in the stratum germinativum. In the stratum granulosum the cytoplasmic enzyme activity tends to be concentrated in the periphery of the cells. In addition, there is a progressive diminution in the amount of phosphatase present from the deeper to the more superficial layers of the stratum granulosum. Little or no enzyme is present in the stratum cor-

neum. Cytoplasmic ribonucleic acid shows a parallel gradient in concentration but is more or less evenly dispersed throughout the cells in fine granules. The cytoplasm of the cells in the stratum germinativum is intensely basophilic. The depth of staining decreases progressively throughout the stratum granulosum. The stratum corneum is completely unstained by toluidine blue at the pH used in the present studies. No mucin is present in any of the cells of the vaginal epithelium in the estrogen treated animal.

As previously stated, the mucification response evoked by treatment with estrogen and progesterone concurrently was somewhat different than that described by Freud. In the present study, only the most superficial layer of epithelial cells has been markedly affected. The cells lining the vaginal lumen are of the cuboidal to low columnar type with basally situated nuclei. The cytoplasm of these cells is intensely stained throughout with basic fuchsin, indicating a high concentration of mucin. In scattered areas of the vaginal epithelium there is a partial mucification of the squamous layers immediately beneath the superficial mucous layer. This divergence from the results of Freud is due probably to differences in experimental conditions and hormone dosage.

The amount and distribution of alkaline phosphatase and cytoplasmic ribonucleic acid in the non-mucified layers of the estrogen-progesterone treated group is comparable to that seen in the stratum germinativum and deeper cells in the stratum granulosum in the estrogen treated animals. The gradient in the concentration of these substances is somewhat less pronounced in the former group. Unlike the cornified layer in the estrogen treated animal, the superficial mucified layer exhibits intense alkaline phosphatase activity in the apical cytoplasm of its cells. It is impossible to determine from our material whether these cells also contain small amounts of ribonucleic acid because of the intense cytoplasmic basophilia due to the presence of mucin.

Discussion. The present experiments show that the injection of estrogen into the ovari-

ectomized mouse is followed by a marked increase in both alkaline phosphatase and cytoplasmic ribonucleic acid. These findings are in substantial agreement with the observations of Jeener.² In addition, it has been shown that progesterone alone does not have this effect. Injection of progesterone concurrently with estrogen neither augments nor diminishes the effect of the estrogen. Such combined treatment results in the eventual mucification rather than cornification of the superficial layers of the epithelium. It is interesting to note that estrogen and progesterone have similar effects on endometrial alkaline phosphatase (Atkinson and Elftman⁶) and cytoplasmic ribonucleic acid (Atkinson, unpublished data).

Relatively little is known concerning the specific functions of ribonucleic acid and phosphatase in cellular metabolism. In a recent extensive review, Caspersson⁷ has presented considerable evidence to support the view that cytoplasmic ribonucleic acid is essential to the synthesis of proteins. The role played by alkaline phosphatase in the hydrolysis of phosphoric esters (*e.g.*, nucleotides, glycoposphates, phospholipids, etc.) makes it potentially important in a wide variety of physiological processes. One possible function of particular interest (Moog,⁸) is the suggestion that phosphatase activity parallels the synthesis of fibrous proteins.

The presence of large amounts of cytoplasmic ribonucleic acid and alkaline phosphatase in the estrogen and estrogen-progesterone stimulated vagina is compatible with the views of Caspersson and Moog in view of the concomitant rapid growth and elaboration of keratin or mucin. The significance of the intracellular distribution of these substances and the gradients in their concentrations is entirely speculative at the present time.

Summary. The amount and distribution of cytoplasmic ribonucleic acid, alkaline phos-

⁶ Atkinson, W. B., and Elftman, H., *Proc. Soc. Exp. Biol. and Med.*, 1946, **62**, 148.

⁷ Caspersson, T., *Symp. Soc. Exp. Biol.*, 1947, **1**, 127.

⁸ Moog, F., *Biol. Rev.*, 1946, **21**, 41.

phatase and mucin was studied in the vaginal epithelium of ovariectomized mice and in castrates injected with estrogen and progesterone. In the untreated castrate the vaginal epithelium contains negligible quantities of all these cellular constituents. Injection of

estrogen, and estrogen with progesterone concurrently, results in a marked increase in both phosphatase and ribonucleic acid. Mucification occurs only when the two hormones act simultaneously. Progesterone alone has no effect on any of the substances studied.

16545

Age and Susceptibility to Convulsions.*

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Although the great susceptibility of children to convulsive disorders is well established, little experimental work has been done on the relationship of age to convulsive reactivity. Froehlich and Mirsky¹ showed that 75 to 100% of 7- to 17-day-old rats convulse when injected with 0.5 to 1.0 mg acid fuchsin per gm subcutaneously. Adult rats however did not show convulsions even after injection with 3 mg per g, but these animals show a high incidence of convulsions after treatment with theophylline. The latter drug seems to increase the permeability of the blood-brain barrier according to experiments of Froehlich and Zak mentioned in this paper (apparently unpublished). This interpretation is supported by Aird^{2,3} and collaborators who found that the percentage of convulsions induced by several convulsive drugs (strychnine, picrotoxin and cocaine) decreased after pretreatment with Brilliant Vital Red or Trypan Red and that the amount of cocaine present in the brain was greatly reduced by the dye although the concentration of cocaine in the blood remained unchanged. Moreover, experiments of Behnsen⁴ on the mouse

and Dölter and Kruse⁵ on human infants have given evidence of a greater permeability of the blood-brain barrier at an early than at a later age. For this reason experiments involving parenteral injection of convulsive drugs cannot be used for the investigation of the question of whether the convulsive reactivity of the brain itself depends on age.

The proper method for studying this question seems to be the application of electrical stimuli adequate to produce convulsions. No experimental studies on normal material seem to have been reported but observations by Watterson⁶ on schizophrenics suggest that the duration of convulsive threshold current increases with age (investigated for the interval of 19 to 49 years). In view of the fact that this study was limited to clinical cases in which the disease process may have contributed to the convulsive reactivity and since the younger age group which is known to be particularly susceptible to convulsions could obviously not be included it was decided to investigate the effect of electrically induced convulsions on rats of varying age.

Method. The experiments were performed on 24 hour fasted Sprague-Dawley rats†

* Aided by a grant from the Office of Naval Research.

¹ Froehlich, A., and Mirsky, I. A., *Arch. Neurol. Psychiat.*, 1942, **47**, 30.

² Aird, R. B., *Arch. Neurol. Psychiat.*, 1939, **42**, 700.

³ Aird, R. B., and Strait, L., *Arch. Neurol. Psychiat.*, 1944, **51**, 54.

⁴ Behnsen, G., *Anat. Anzeiger*, 1926, **61**; *Erg. H.*, 179, and *Z. Zellforschung*, 1927, **4**, 515.

⁵ Dölter and Kruse cf. Gellhorn, E., *Das Permeabilitätsproblem*, Berlin (Springer), 1929.

⁶ Watterson, D. J., *Neurol. Neurosurg. Psychiat.*, 1945, **8**, 121.

TABLE I.
Age and Convulsive Reaction to Electroshock.
I. Current of 24 ma for 0.8 sec.

No. of rats	Average		No. of convulsant rats					% of convulsant rats
	Wt, g	Age, wks	++++*	+++	++	+	±	
10	52	4	10	0	0	0	0	100
27	72	7	26	1	0	0	0	100
29	120	10	15	8	4	2	0	100
9	164	18	1	0	3	1	4	44
30	244	24	0	0	1	5	24	20
18	282	50	0	0	0	1	17	6
II. Current of 27 ma for 0.8 sec.								
37	250	37	5	4	0	3	25	32
24	342	58	1	0	0	1	22	8
15	360	76	0	0	0	1	14	7
III. Current of 29 ma for 0.8 sec.								
12	228	25	3	3	2	3	1	92
11	233	68	1	2	1	1	6	46

* +++++ = tonic-clonic convulsions.
 ++++ = clonic convulsions > 10 sec.
 +++ = " " 6 to 10 sec.
 ++ = " " 2 to 5 sec.
 + = " "
 ± = no convulsions.

varying in age between several weeks to more than 2 years. A shock of 0.4 second duration was applied to both ears with a stimulator† which delivered a 60 cycle square stimulus. The output current to the animal is independent of the resistance of the animal and the electrodes. It can be applied for any interval by a manual switch or an electronic photo timer. The stimulating current is derived from one-half of the 700 volt secondary of a conventional radio power transformer applied to the animal in series with the plate of a 6F6 radio tube whose screen voltage is held constant by a voltage regulator tube. Since the plate current of a pentode with a constant screen voltage is independent of its voltage over a wide range, the current through the animal is independent of its resistance or the contact resistance of the electrodes. No electrode paste need be used. The waveform approaches a square wave since the 6F6 tube rectifies the 60 cycle sine wave and because of its constant current characteristics the current rises almost instantly to the predetermined value and remains there for approximately 1/120 sec. when it drops rapidly to zero. The cur-

rent is measured by placing a d.c. milliammeter across the output terminals and adjusting the cathode resistor of the 6F6 tube until the desired current is obtained. If a square wave of 180° duration is assumed the meter reading is approximately ½ the peak current. The time of stimulation was controlled by placing the relay contacts of an electronic photo timer across the output terminals in such a manner as to act as a short except during stimulation.

Results. The first part of Table I shows that the incidence of convulsions decreased as the average weight increases from 52 to 282 g corresponding to an age ranging from 4 to 50 weeks. A current of 24 ma induced convulsions in all rats up to an average weight of 120 g but led only to 6% convulsions when rats of a mean weight of 282 g are used. Moreover the table shows that the severity of the convulsions declined with increasing age. The convulsive stimulus produced clonic-tonic convulsions in 100% of the younger and 0% of the older group listed in Section I. The convulsions were exclusively of the clonic type in the latter group. These results were confirmed in a second series of experiments in which older rats were subjected to a stimulus of 27 ma. Here again a decline in convulsive respon-

† Unanesthetized. The type of electroshock used was found to be painless in man.

‡ Constructed by Mr. John Killen.

TABLE II.
The Influence of Eserine on the Effect of Electroshock.

No. of rats	Current	Number of convulsant animals					% of maximally convulsant rats	
		Without eserine	A %	With eserine*	B %	$\frac{B-A}{A} \times 100$	Without eserine	With eserine
87	20 ma†	19	22				5	
87	20 ma			27	31	+ 42%		4
63	23 ma	17	27				8	
74	23 ma			38	51	+ 123%		25
48	25 ma	5	10				4	
47	25 ma			9	19	+ 80%		12

* 1 mg/kg subcutaneously.

† Alternating current, 60 sec., applied for 0.4 seconds.

siveness resulted from increased weight and age.

A final group shows the convulsive reactivity of adult rats of similar weight but of different age. All rats received their food (dog chow) ad libitum, but the rats of 1½ years of age and older had the tendency to lose weight. They could therefore easily be matched in weight by a younger group. In this experiment (Table I, pt. 3) adult rats averaging 25 and 68 weeks of age respectively were shocked with 29 ma. The results show clearly that age is a decisive factor since incidence and severity of convulsions decrease with increasing age although weight differences are absent.

Discussion. Although the threshold for convulsions of individual rats was not determined the fact that a current of 24 ma causes severe tonic-clonic convulsions in 100% of the young rats and brief clonic convulsions in only 6% of rats of an average age of 50 weeks indicates that the convulsive threshold for electrical currents increases with increasing age. This phenomenon is not confined to the first months of life until maturity is reached but applies to rats of more than 6 months of age. However the data are inadequate to answer the question as to whether the decline in convulsive reactivity progresses to old age.

Since the blood-brain barrier is not involved in these experiments the convulsive reactivity of the brain itself seems to vary with increasing age. As was shown in a previous paper⁷ the convulsive response to a

large number of convulsant drugs is increased through pretreatment with mecholyl or eserine under conditions of topical application to the cortex. This may suggest that the effect dealt with in this paper is related to the acetylcholine metabolism of the brain. The increasing concentration of brain cholinesterase (Nachmansohn,⁸ Welsh, and Hyde⁹) and the diminishing sensitivity of rats to DFP with increasing age (Freedman and Himwich¹⁰) are compatible with this interpretation. In order to clarify further the relation of acetylcholine metabolism to convulsive activity in general, experiments were performed on the effect of eserine on the reactivity of rats to electrically induced convulsions. Eserine administered intravenously is known to alter the pulse rate as blood pressure falls, and such circulatory changes are known to modify greatly the convulsive activity (Gellhorn *et alii*¹¹). The subcutaneous injection of eserine, however, sensitizes the animal to electrically induced convulsions (Table II) but does not alter the pulse rate (Table III). This suggests

⁷ Hyde, J., Beckett, S., and Gellhorn, E., in press.

⁸ Nachmansohn, D., *Bull. Soc. Chem. Biol.*, 1939, **21**, 761.

⁹ Welsh, J. H., and Hyde, J. E., *J. Neurophysiol.*, 1944, **7**, 41.

¹⁰ Freedman, A. M., and Himwich, H. E., *Symposium on Military Physiology, Digest series No. 4*, 1947.

¹¹ Gellhorn, E., Yesinick, L., and Kessler, M., *Am. J. Physiol.*, 1942, **137**, 396.

TABLE III.
Effect of Eserine on Pulse Rate of Rats.*

No.	Wt in g	Pulse rate/min.	
		Before	30' after es.
1	250	324	321
2	210	345	336
3	230	372	388
4	280	480	479

* Eserine sulfate (1 mg/kg) was injected subcutaneously and the effect on the pulse was determined by recording the EKG with an Offner oscillograph.

that eserine directly sensitizes the brain substance to electroshock, quite apart from changes in reactivity resulting from circulatory effects.

There seems to be no good evidence to relate the age dependent susceptibility of rats to convulsions to the water content of the brain (Yannet and Darrow¹²). Donaldson¹³ notes that the water content of the brain of the albino rat although declining from the 1st to the 50th day remains constant from there on to the age of 300 days

¹² Yannet, H., and Darrow, D. C., *J. Biol. Chem.*, 1938, **123**, 295.

¹³ Donaldson, H. H., *The rat, Memoirs of the Wistar Inst. of Anat. and Biol.*, No. 6, Philadelphia, 1924.

and that no variation occurs in Norway rats varying in weight between 195 and 460 g. However our experiments show clearly that within these ranges of age and weight convulsive reactivity changes greatly.

Finally attention is called to the fact that the excitability of the sympathetic centers declines with increasing age (Safford and Gellhorn¹⁴). The relation of this fact to the topic of the present paper remains to be determined.

Summary. Experiments on rats varying in age between 4 weeks and more than 1½ years show that the incidence and severity of convulsions induced by application of an electrical current to the head declines with increasing age. This decline is not confined to the period preceding sexual maturity. Comparison of adult rats of different age but similar weight shows that the susceptibility to convulsions is related to age and not solely to weight. Furthermore it is shown that eserine administered subcutaneously increases the susceptibility to electrically induced convulsions without altering the circulation.

¹⁴ Safford, H., and Gellhorn, E., *Proc. Soc. Exp. Biol. and Med.*, 1945, **60**, 98.

16546

A Comparison of Cardiac Outputs, Determined by Direct Fick and Pressure Pulse Methods.

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A method of determining cardiac output that is more rapid than the standard procedures, and with less dependence on skilled technical help has long been needed. With the publication by Hamilton and Remington¹ of a method for determining stroke volume from a pressure pulse this need has

been met if their results are reproducible by others. In addition, their method has the added advantage, for the pharmacologist, of being able to follow the actions of a drug on the output from stroke to stroke.

Using the dye method as their basis of comparison, they found a correlation of $r = +0.994$ with an average variation between the two measurements of $\pm 8.2\%$

¹ Hamilton, W. F., and Remington, J. W., *Am. J. Physiol.*, 1947, **148**, 14.

TABLE I.
Comparison of Cardiac Output by Direct Fick and Pressure Pulse Methods.

Dog	Procedure	Direct Fick*	Pressure pulses*	% difference
D.F. 2	Aranthol, 5 mg/kg	2,500	3,000	+20
D.F. 3	Priscol, 1 mg/kg	1,550	1,524	— 1.6
	Oenethyl, 5 mg/kg	3,120	3,400	+ 8.1
D.F. 4	Priscol, 1 mg/kg	2,300	2,567	+11.6
D.F. 5	Normal	5,350	4,520	—15.5
	Hemorrhage	3,950	3,400	—13.9
D.F. 6	Normal	2,910	2,477	—14.8
	Hemorrhage	1,210	1,489	+23.0
D.F. 7	Hemorrhage	2,300	1,950	—15.2
	"	1,350	1,145	—15.1
D.F. 8	Normal	1,350	1,306	— 3.2
	Aranthol, 10 mg/kg	4,810	4,262	—11.3
	½ hr after	1,620	1,661	+ 2.5
D.F. 9	Normal	2,160	1,944	—10.0
	Priscol, 1 mg/kg	2,300	2,700	+17.4

* cc/min.

with a range of +35 to —13%. While the dye method has been shown to give comparable to the Fick method² within $\pm 10\%$, it seemed essential that a comparison be made between the Fick and the pressure pulse method not only to compare the accuracy of the pressure pulse method but to check our own calculations of cardiac output by that method.

Methods. A total of 8 dogs were used and 15 outputs were determined under a variety of conditions. The dogs were anesthetized with sodium barbital 300 mg/kg administered orally or intraperitoneally. Pressure pulses were obtained by means of a Hamilton manometer³ of adequate frequency. A No. 9 ureteral catheter was passed down the right jugular to the right ventricle and the position verified by fluoroscopy. A slow, heparinized saline drip was maintained through the catheter. Oxygen consumption was measured by connecting the dog to a McKesson Recording Metabolor, with a tracheal cannula. Blood samples were taken

under oil simultaneously from the right ventricle and a femoral artery and promptly iced. Oxygen was determined by the method of Van Slyke and Neill.⁴ Pressure pulses were recorded simultaneously with the blood samples. Under certain circumstances marked arrhythmia and a variety of pulse forms occurred. Depending on the frequency, some of each type were calculated and an average figure was obtained.

It was thought desirable to have as wide a range of outputs as possible. This was accomplished by hemorrhaging and the use of the following drugs: 2-methylamino heptane (Oenethyl), 2-methylamino-6-hydroxy-6-methyl heptane (Aranthol), and benzylimidazoline hydrochloride (Priscol).^{*} By these means heart rates from 80-260 per minute, systolic pressures from 95-352 and diastolic from 65-220 mm Hg were obtained. The data given by Hamilton and Remington for diastolic pressures does not extend to 220 mm Hg; in this case we extrapolated

⁴ Van Slyke, D. D., and Neill, J. M., *J. Biol. Chem.*, 1941, **138**, 535.

² Moore, J. W., Kinsman, J. M., Hamilton, W. F., and Spurling, R. G., *Am. J. Physiol.*, 1929, **89**, 331.

³ Hamilton, W. F., Brewer, G., and Brotman, I., *Am. J. Physiol.*, 1934, **107**, 427.

* The authors wish to thank the following for supplying the drugs: Oenethyl and Aranthol, Bilhuber-Knoll Corp., and Ciba Pharmaceutical Products, Inc. (Priscol).

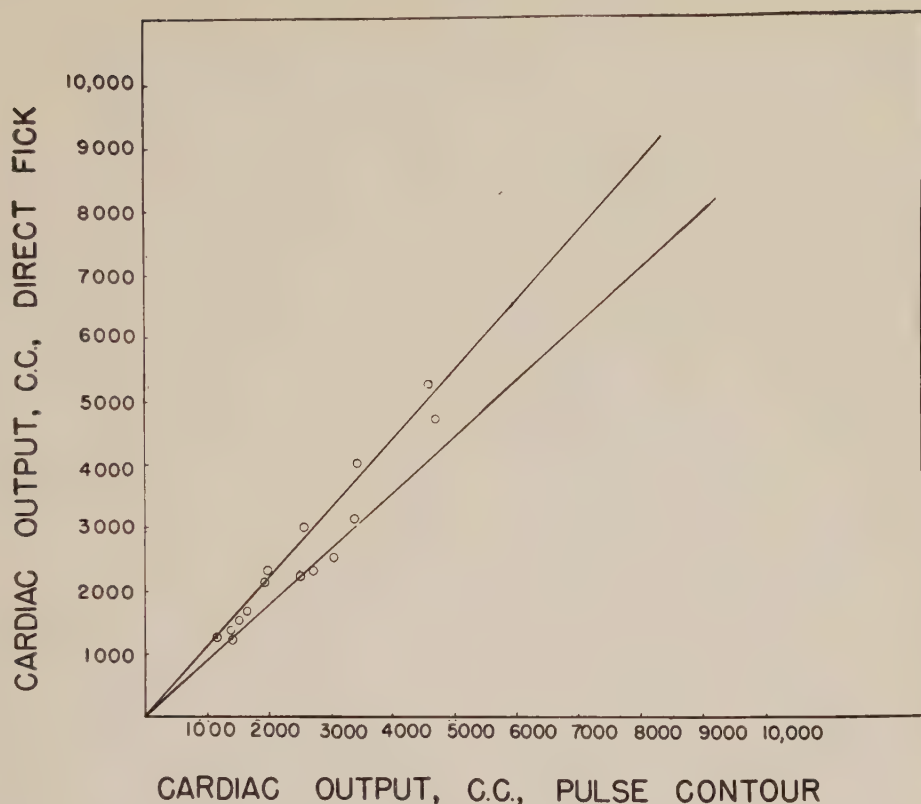


FIG. 1.

Relationship between cardiac output determined simultaneously by the direct Fick and pressure pulse methods. The lines represent a deviation of $\pm 10\%$ from a perfect correlation.

with satisfactory results.

Results and Conclusions. The determination of cardiac output by the pressure pulse method agrees well with the direct Fick method. The average variation between the 2 measurements is $\pm 12.2\%$ with a range

from $\pm 23\%$ to -15.5% . The coefficient of correlation, corrected for small samples, is $r = + 0.936$. Directional changes in cardiac output can be conveniently followed by the pressure pulse method.

Production of a Less Sensitive Rapid Antigen for Diagnosis of *Brucella* Infections.

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(Introduced by Herman C. Lichstein.)

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The production of oversensitized antigens for use in diagnosis of *Brucella* infections has created a problem in the interpretation of agglutination tests. According to Huddleson,¹ the presence of dissolved agar in dilutions as high as 1-50,000 in the antigen can cause nonspecific agglutination of the organisms. Tallman² employed cellophane sheets over Bordet-Gengou agar for the cultivation of *Hemophilus pertussis* with considerable success in ridding suspensions of blood cells and possibly of dissolved agar. Later, Moore and Mitchell³ suggested the use of a dialysing membrane layered over broth absorbed in cotton. Growth was obtained in this manner and used in the production of a less sensitive antigen. Harmsen and Kolff⁴ also employed cellophane as a dialysing membrane for growth of bacteria nourished by fluid on the opposite side. They pointed out that the membranes were impermeable to bacteria and viruses, but allowed passage of water and solutes, except those of very large molecular weight.

The present paper is concerned with a cultural method designed to eliminate oversensitivity of rapid antigen by using a collodion dialysing membrane in conjunction with agar plates as described.

Method and Equipment. A BAI strain of *Brucella abortus* exhibiting normal agglutinability was employed throughout. The organisms were grown on a sterile collodion mem-

brane* superimposed on the surface of an agar plate. The collodion sheets were cut so as to give a circular sheet of approximately 130 cm in diameter to fit over a large Petri dish. Each sheet was layered between a piece of flat wrapping paper backed by cardboard in order to maintain the flatness of the membrane and sterilized by autoclaving at 121° C, for 30 minutes.

The medium used was Bacto-tryptose agar. To obtain larger areas of growth, the medium was poured into sterile 150 cm Petri dishes and allowed to solidify. The collodion membrane was then placed on the surface of the agar by using a pair of sterile forceps, flaming after each plating. A tendency for the membrane to curl was noted. This objectionable feature was rectified by allowing plates to stand inverted in the refrigerator for several hours after which time a smooth surface was obtained. Extreme caution was taken at the time of placing the membrane on the agar surfaces as possibilities for contamination during this procedure were very great.

A 24 hour culture of *Brucella abortus* was used. To this culture was added 5 ml of tryptose broth and the growth removed from

¹ Huddleson, I. Forrest, *Brucellosis in Man and Animal*, 1939, 192.

² Tallman, A. W., *Personal Communication*, 1942.

³ Moore, T., and Mitchell, C. H., *J. Am. Vet. M. A.*, 1945, **107**, 226.

⁴ Harmsen, G. W., and Kolff, W. J., *Science*, 1947, **105**, 582.

* There are two types of collodion membrane that might be used. The first is covered with a fine coating of plastic film. This coating prevents the free passage of nutritional fluids through the membrane and thus must be removed before use. This can be done readily by boiling for several minutes in a 5% solution of bicarbonate. Commercial cellophane is permeated with oil, which renders it unsatisfactory. The other type of cellophane is that which is free from either a coating film or oil and readily permits free passage of nutrients. This type was used throughout the work.

the slant with the aid of a sterile inoculating needle. A sterile cotton swab dipped into the culture was used for the inoculation of each plate by more or less "painting" the surface of the membrane. The pressure exerted on the swab was also useful in removing any air bubbles that might have been entrapped under the membrane. Again extreme care was exercised to prevent possible contamination.

The plates were then inverted and incubated at 37° C for 4-5 days. The cells were harvested by scraping the growth with a sterile spatula and were deposited into a previously weighed flask. Ordinarily about 15% of the plates were contaminated and therefore discarded. Others showed slight contamination under the collodion membrane or had only one or two contaminating colonies on the surface of the membrane. When plates of this nature were used, care was taken in removing the growth so as not to pick up contaminating colonies. After the growth was removed, the flask was reweighed to ascertain the amount of bacterial mass present. To the flask was added enough suspending solution (12% NaCl-20% glycerine and 0.5% phenol) to make a suspension of cells that was between 18 to 20% by net weight. To this suspension was added crystal violet, and brilliant green in a dilution of 1:25,000, to inhibit growth of possible contaminants. The cells were killed by heating in a water bath at 60° C for one to 2 hours. The antigen was then filtered through a sterile cotton filter and dispensed into clean dropping bottles. Sensitivity of the antigen was titered according to the method of Huddleson.⁵

Results. Three types of antigen, namely rapid antigen and test tube antigen as described by Huddleson⁶ and the collodion membrane rapid antigen described in this paper were used in each test. All agglutination tests were performed in accordance with the standard procedure recommended by Huddleson.⁷

TABLE I.
A Selective Number of Samples Showing the Type of Agglutination with Each Antigen.

Sample	1:25			1:50			1:100			1:200		
	Regular rapid	Collodion rapid	Test tube	Regular rapid	Collodion rapid	Test tube	Regular rapid	Collodion rapid	Test tube	Regular rapid	Collodion rapid	Test tube
1	+++	++	++	++	++	++	++	++	++	++	++	++
2	+++	++	++	++	++	++	++	++	++	++	++	++
3	+++	++	++	++	++	++	++	++	++	++	++	++
4	+++	++	++	++	++	++	++	++	++	++	++	++
5	+++	++	++	++	++	++	++	++	++	++	++	++
6	+++	++	++	++	++	++	++	++	++	++	++	++
7	+++	++	++	++	++	++	++	++	++	++	++	++
8	+++	++	++	++	++	++	++	++	++	++	++	++
9	+++	++	++	++	++	++	++	++	++	++	++	++
10	+++	++	++	++	++	++	++	++	++	++	++	++
Positive.	+	+	+	+	+	+	+	+	+	+	+	+
Negative.	-	-	-	-	-	-	-	-	-	-	-	-
Incomplete positive.	±	±	±	±	±	±	±	±	±	±	±	±

⁵ Huddleson, I. Forrest, *Op. Cit.*

⁶ *Ibid.*

⁷ *Ibid.*

TABLE II.
Number of Tubes Showing Complete Agglutination with Each Antigen.

Antigen employed	Dilution			
	1:25	1:50	1:100	1:200
Regular rapid antigen	19	19	11	30
Test tube antigen	16	11	8	29
Collodion rapid antigen	16	9	3	30

Of the 301 blood samples tested, using the three sets of antigens, 38% of the specimens gave evidence of agglutination. This agglutination ranged from very slight in the 1-25 dilution of serum to complete agglutination in dilutions to 1-200. Actually only 15% were reported as "positive." Table I lists a selective number of samples and the types of reaction given with each antigen. Table II shows the number of reactions called "positives" in each dilution. The collodion membrane rapid antigen proved to be less sensitive in 65 of the samples when compared with regular rapid antigen, and was less sensitive than the test tube antigen in 19 samples. In 3 samples collodion membrane rapid antigen was more sensitive than regular rapid antigen, and in 13 samples more sensitive than test tube antigen. All 3 antigens were in agreement in 32 of the samples. In 3 samples only collodion membrane rapid antigen and the regular rapid agreed, whereas in 37 samples only collodion membrane rapid antigen and test tube antigen were in agreement. In 54 samples regular rapid antigen showed more sensitivity than did test tube antigen.

Discussion. Rapid antigen obtained from growth on collodion membranes, appears to be superior to regular rapid antigen as indicated by closer agreement with test tube antigen reactions, particularly in dilutions of serum

below 1:200. References to the sensitivity of rapid antigen imply that the presence of dissolved agar may be partially responsible for oversensitivity. We made no test for permeability of the collodion membrane to dissolved agar, but the fact that collodion membrane rapid antigen gave more reliable results than regular antigen indicates that the factor responsible for oversensitivity was not present in sufficient amount to be significant.

Therefore, the use of a collodion membrane as a mechanism for eliminating factors responsible for oversensitization of antigens appears worthy of application to the production of *Brucella* and other rapid antigens showing a tendency to oversensitivity.

Summary. The preparation of a rapid antigen for use in the agglutination reaction for the diagnosis of *Brucella* infection is described. The essential difference between this antigen and former preparations is the growth of the organism on collodion membranes layered over nutrient agar.

When compared with regular rapid antigen, the collodion membrane rapid antigen showed markedly less sensitivity in serum dilutions of 1:100 or less. The collodion membrane rapid antigen and test tube antigen were more frequently in agreement than test tube antigen and regular rapid antigen.

Intravenous Glucose Transformation During Severe Muscular Exercise in Normal Dogs.

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Intravenous glucose is better assimilated during work than levulose, galactose or maltose,¹ when injected at a low rate. It was therefore thought advisable to examine the laws of its utilization in animals undergoing severe exercise (climbing a treadmill), thus complementing the similar study which has already been done for normal resting animals.² The purpose of the present research was to test the power of the body to transform this most utilizable foodstuff throughout the whole range of rates of constant supply and of its concentrations in the body fluids, obtainable in a living, working animal. As may be seen from the recent reviews,^{3,4,5,6} the knowledge on that subject is still nonexistent.

Dogs, kept on a constant high-carbohydrate diet, were made to perform work, during 5 hours, at two rates: either 1000 or 1250 kg/hr/kg body wt (causing a 4-6-fold increase in basal metabolism) and were injected with glucose at constant rates which varied from 20-230 g/sqm/hour (1-12 g/kg/hour)⁸ in different experiments, with proper regulation of water balance. When the animals performed work with saline added instead of glucose the level of blood glucose dropped a few mg%. Lactic acid in the blood increased above the resting level about 10 mg% and increased in the urine moderately. This intensity of work led therefore to some discharge of lactate from the contracting muscles.

Although utilization of injected glucose

rose greatly during work, the initial phase of lower assimilation⁷ was not abolished.* On the contrary it was relatively more marked and protracted. Still with an injection rate of 4 g/kg/hour, glucose elimination vanished towards the end of the experiment, whereas in resting animals even with a rate of 2 g/kg/hour, such an improvement of glucose transformation could never be achieved.²

The average values of glucose utilized* when plotted as ordinates against the increase of blood glucose concentration over the normal pre-injection level, as abscissa, showed an exponential rise of utilization with the rise of blood glucose, up to about 1000 mg%. At this concentration a peak of utilization was reached: at 1000 kg/hr/kg body wt, 107 g/sq m/hour were assimilated;⁸ at 1260 kg/hr/kg body wt, (128 g/sq m/hour.) The highest value obtained with the latter rate of work was about 150 g/sq m/hour (= 14 times the basal heat production = 6 g/kg/hour). In resting animals on the average the peak of transformation was reached at 750 mg% and amounted to 79 g/sq m/hour. In accordance with a previous communication, a shift to a higher level of utilization was accompanied by a shift to a higher blood glucose concentration.⁹ Both may depend to some extent on the rise of body temperature¹⁰ as a consequence of severe work.

No more increment in utilization was ob-

⁷ Wierzechowski, M., *Biochem. Z.*, 1931, **237**, 103.

* Computed from blood and urine glucose curves, using the figure of 20% of body weight as body water, in which glucose was distributed.

⁸ Cowgill, G. R., and Drabkin, D. E., *Am. J. Physiol.*, 1927, **81**, 36.

⁹ Wierzechowski, M., *Abstracts of Comm. XVII International Physiological Congress, Oxford*, p. 256.

¹⁰ Wierzechowski, M., *Fed. Proc.*, 1948, **7**, 134.

¹ Wierzechowski, M., Chmielewski, T., Gostyńska, A., and Fiszal, H., *Compt. Rend. Soc. de Biol.*, 1935, **119**, 433.

² Wierzechowski, M., *J. Physiol.*, 1936, **87**, 311.

³ Dill, D. B., *Physiol. Rev.*, 1936, **16**, 263.

⁴ Atzler, E., *Erg. Physiol.*, 1939, **41**, 164.

⁵ Gemmill, C. L., *Physiol. Rev.*, 1942, **22**, 32.

⁶ Keys, A., *Fed. Proc.*, 1943, **2**, 164.

tained when the blood glucose concentration was further raised. The whole surplus of glucose was rejected in the urine, presenting also during work the phenomena of overflow diabetes described for resting animals.² If 2500-3000 mg% of blood glucose were reached some extra amount of work was imposed upon the muscles by convulsive phenomena originating from stimulation of the segmental, motor nuclei across the whole neuraxis¹¹ and seemed to give some transitory further increase of glucose retention, before final weakness and failure of circulation took place.

Up to 1000 mg% of blood glucose, blood lactate was somewhat higher at work than at rest. At 1000 mg% a peak of lactate concentration was reached (35 mg% above basal value) almost exactly equal to the one observed for a resting animal. This concentration persisted up to 2500 mg% of

blood glucose, when convulsive phenomena began to augment the blood lactate formation intensely. In the urine at each value of blood glucose, lactate excretion was greater at work than at rest, the peak being reached at 1600 mg% of blood glucose; then the elimination decreased both at work and at rest with deterioration of circulation in the kidneys. It is evident that each value of blood glucose at work corresponded to a higher level of glucose transformation than at rest. Therefore, as expected, lactate, the rejection of which into the blood stream parallels glucose transformation, is formed in larger amounts during work at identical blood glucose levels, than at rest. The work itself also may contribute to its increased passage into the bloodstream.

Conclusion. Work does not increase to an unlimited extent the transformation of glucose supplied to the muscles in highest amounts. Its utilization at work, although even almost doubled, is governed by laws similar to those at rest.

¹¹ Wierzechowski, M., Toczyski, T., and Sysa, J., *Am. J. Med. Sc.*, 1948, **215**, 108.

16549

Stimulation of Gastric Secretion by Urticariogenic Wetting Agent (Tween 20) and Its Inhibition by Benadryl.

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(With the technical assistance of James Harris.)

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The synthetic wetting agent Tween 20 (Atlas Powder Co.), which is a polyoxy-alkylene derivative of sorbitan monolaurate, has recently been shown to be capable of producing generalized urticaria when injected intravenously in dogs.¹

Several investigators^{2,3} have found that when whealing of the skin is produced by mechanical or thermal injury in the human, gastric acid secretion is stimulated. The

usual interpretation of this phenomenon is that injury to the skin causes the epidermal cells to release either histamine or a histamine-like substance (H-substance). This substance acts locally at the site of release to produce the whealing reaction and some of it is carried into the general circulation where it produces systemic effects similar to those produced by the injection of histamine, including stimulation of acid secretion by the stomach.

The present study was undertaken in order to determine whether the urticaria which occurs in dogs when Tween 20 is injected is accompanied by stimulation of gastric se-

¹ Ivy, A. C., Tanturi, C.A., Hernandez, R., and Baroso, E., *Arch. Derm.*, in press.

² Horton, B. T., Brown, G. E., and Roth, G. M., *J.A.M.A.*, 1936, **107**, 1263.

³ Kalk, H., *Klin. Wschr.*, 1929, **8**, 64.

TABLE I.

The Effect of Tween 20 (0.8 mg/kg Intravenously) on Gastric Secretion and Urticarial Reaction in Dogs, With and Without Benadryl (10 mg/kg Intravenously) One-half Hour Before the Tween 20.

Dog No.	Tween 20 without Benadryl			Tween 20 after Benadryl		
	Urticaria ++ + 0	Basal secretion Free HCl output (mg/hr)	Gastric secretion Free HCl output (mg/2 hr)	Urticaria ++ + 0	Basal secretion Free HCl output (mg/hr)	Gastric secretion Free HCl output (mg/2 hr)
1	x	0	58.8	x	0	12.1
1	x	1.9	130.9	x	0	6.2
2	x	0	6.9	x	0.2	0
2	x	0	9.4	x	0	0
2	x	0	25.6	x	0	0
3	x	0.8	37.2	x	0	6.5
3	x	0	19.6	x	0	0
4	x	0	18.1	x	1.8	34.0
4	x	0	14.2	x	0	58.3
5	x	2.1	71.0	x	0	0
5	x	0	14.9	x	0	0
6	x	0	19.6	x	0	6.4
6	x	0	92.7	x	2.0	16.3
6	x	0.6	32.4	x	0	14.5
7	x	0	31.0	x	0	5.5
7	x	0	15.7	x	0	24.9
8	x	0.9	25.8	x	0.4	8.3
8	x	0	16.9	x	0	35.8
Total or avg	18 0 0	0.4	35.5	2 9 7	0.3	12.7

++ Marked urticarial response.

+ Slight " "

0 No " "

Statistical analysis (method of paired comparison, Snedecor,⁵ p. 84): mean difference = 22.8, standard error of mean difference = ± 10 , $t = 2.29$, $p \pm 0.04$.

cretion. Since we found that gastric secretion was stimulated, we proceeded to study the effect of the antihistaminic drug benadryl (beta-dimethylaminoethyl benzhydryl ether hydrochloride) on this stimulation.

Procedure. Eight dogs with pouches of the entire stomach were used. Tween 20 was injected intravenously in a dose of 0.8 mg per kilo of body weight, using a 1% aqueous solution. In one half of the experiments, a 1% aqueous solution of benadryl was injected intravenously in a dose of 10 mg per kilo one half hour before giving the Tween 20. Gastric juice was collected every half hour for 2 periods preceding the injection of Tween 20 and for 4 periods after the injection. The volume of each sample was measured and it was then titrated with 0.027 N NaOH, using *p*-dimethylaminoazobenzene as an indicator. Observations were made on the severity of the urticarial reaction in each

instance. Dogs were not used oftener than once every 5 days for these experiments.

Results. The results are summarized in Table I. Tween 20 alone produced marked urticaria and stimulated gastric secretion in each of the 18 tests on the 8 dogs. The gastric secretory response begins in 15 to 45 minutes following the injection, reaches its peak during the second half-hour period, and has returned to the basal level by the end of 2 hours. Urticaria begins to occur 3 to 5 minutes after the injection, reaches a maximum in 10 to 15 minutes, and has usually subsided completely within 30 minutes after injection. When Tween 20 was injected one-half hour after benadryl had been given, both urticaria and gastric secretion were either completely abolished or definitely diminished in 14 out of 18 tests on 7 of the dogs. In the remaining 4 tests on 3 dogs, urticaria was only questionably or moderately reduced and gastric secretion was the same or slightly greater than in the tests

⁵ Snedecor, G. W., *Statistical Methods*, 4th ed., Iowa State College Press, Ames, Iowa, 1946.

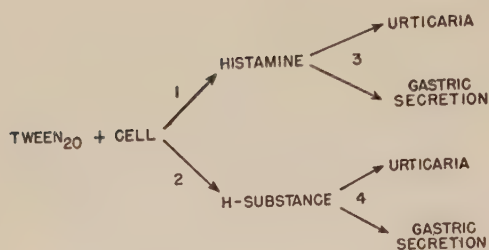


FIG. 1.

with Tween 20 alone. Statistical analysis reveals that the difference between the results with and without benadryl could be expected to occur by chance variation due to random sampling error in less than 4 out of 100 similar experiments.

Discussion. The simplest explanation that could be offered for the results we have obtained is that Tween 20 causes histamine to be released and that benadryl counteracts both the urticariogenic and gastric secretory effects of the released histamine. However, we⁴ have previously shown that benadryl does not inhibit the gastric secretory response to histamine in dogs. Inasmuch as a dose of benadryl of only 5 mg/kg subcutaneously was used in these earlier studies, we have repeated the experiment using 10 mg/kg intravenously. In 4 tests on 4 dogs, this higher dose of benadryl also failed to depress the secretory response to 0.0125 mg of histamine dihydrochloride given every 10 minutes. The average secretory response when this dose of histamine is used is comparable to the average secretory response to the dose of Tween 20 used in these studies. Therefore, inasmuch as benadryl does not counteract the action of histamine on the gastric glands, another ex-

planation must be sought for the depression of the secretion evoked by Tween 20.

Fig. 1 is a simple schematic representation of some of the possible mechanisms by which Tween 20 may act and the points at which benadryl may counteract it. According to this analysis, if Tween 20 releases histamine, then benadryl must act at 1 to prevent the release of this histamine. This is so because once the histamine is released, benadryl cannot counteract its action on gastric secretion; that is, it cannot act at the point designated 3 in the diagram. On the other hand, if the gastric secretion produced by Tween 20 is due to a histamine-like substance, then benadryl may, either block its formation or block its action on the gastric glands (points 2 or 4 in the diagram).

In any case, it is significant that the action of benadryl in counteracting gastric secretion stimulated by Tween 20 cannot be accounted for on the basis of its known pharmacological properties. This suggests that benadryl may inhibit anaphylactic and anaphylactoid reactions by some mechanism in addition to blocking the action of histamine on effector cells.

Summary. 1. Tween 20 produces urticaria and stimulates gastric secretion when injected intravenously in a dose of 0.8 mg/kg in dogs with gastric pouches.

2. Pretreatment with benadryl (10 mg/kg intravenously) prevents or reduces the urticariogenic and secretory effects of Tween 20 in most instances.

3. Inasmuch as benadryl does not inhibit histamine-stimulated gastric secretion, the mode of action of benadryl in counteracting Tween 20 stimulated secretion must involve another mechanism.

⁴ Sangster, W., Grossman, M. I., and Ivy, A. C., *Gastroenterology*, 1946, **6**, 436.

16550

Effects of a New Congener of Dibenamine on the Actions of Sympathomimetic Amines.*

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(Introduced by H. B. van Dyke.)

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Since the announcement by Nickerson and Goodman^{1,2} of the adrenolytic and sympatholytic potency of some compounds of the nitrogen mustard series, several groups of workers have published reports of various aspects of the autonomic pharmacology of such compounds. A logical extension of the discovery of the adrenolytic properties, *viz.*, an examination of the capacity of such compounds to modify the sympathomimetic effects of substances other than epinephrine itself, was undertaken and is the subject of this report. The experiments were performed with N-benzyl-N- β -phenylisopropyl- β -chloroethylamine HCl (drug 194),[†] a substance closely related to Dibenamine. Wells and Rall³ recently published an abstract of work similar in purpose.

Methods All the experiments were performed with cats (1-5 kg) anesthetized with either sodium pentobarbital (30 mg/kg intraperitoneally), or Dial with urethane (0.5 ml/kg intraperitoneally). One or 2 mg of atropine sulfate was administered intramuscularly or subcutaneously. Carotid blood pressure was recorded, by a constant perfusion technique, with a mercury manometer.⁴ In some experiments nictitating membrane contractions were registered. Con-

tractions produced by faradic stimulation of the preganglionic cervical sympathetic nerve were compared with drug-induced contractions.

Drug 194, in concentrations of about 2.5×10^{-3} M, was infused from a burette into a femoral vein during a period of about 20-60 minutes. Immediately before use, the drug was diluted from a stock solution of 0.1 M 194 in 95% alcohol (made 0.05 N acid with conc. H_2SO_4). The sympathomimetic amines were injected rapidly intravenously from a 1 or 2 ml tuberculin syringe. These were diluted from stock solutions containing 0.1% $\text{Na}_2\text{S}_2\text{O}_5$ and 0.5% chloretone. All stock solutions were kept in a refrigerator, and, with the exception of one solution of racemic norepinephrine, did not deteriorate. All drugs having an asymmetric carbon atom were used as racemic mixtures—with the exception of epinephrine, whose levo-isomer was used.

The type of experiment which could be performed depended upon the sympathomimetic amine to be used. Catecholic sympathomimetic amines do not cause tachyphylaxis,⁵ so that repeated control doses were administered prior to the injection of the sympatholytic. A single dose of non-catecholic amine was injected after the pressor response to a test dose of 6 $\mu\text{g}/\text{kg}$ of epinephrine had been reversed by 194. The dose of non-catecholic amine was chosen as that which had a pressor-potency equal to or greater than that of 6 $\mu\text{g}/\text{kg}$ of epinephrine injected intravenously.

Results. I. Blood Pressure. A. Catecholic pressor amines. Since tachyphylaxis does not occur with multiple injections of these compounds, small doses (*i.e.* $\leq 10 \mu\text{gm}/\text{kg}$)

* This investigation was supported by a grant from the Smith, Kline and French Laboratories.

¹ Nickerson, M., and Goodman, L. S., *Proc. Am. Fed. Clin. Research*, 1945, **2**, 109.

² Nickerson, M., and Goodman, L. S., *Fed. Proc.*, 1946, **5**, 194.

[†] This drug was furnished by the Smith, Kline and French Laboratories. It will be referred to as 194, the code number of the supplier.

³ Wells, J. A., and Rall, D. P., *Fed. Proc.*, 1948, **7**, 264.

⁴ Trendelenburg, P., *Pflüger's arch. f. d. ges. Physiol.*, 1924, **203**, 413.

⁵ Beyer, K. H., *Physiol. Revs.*, 1946, **26**, 169.

TABLE I.
 Experiments with Catecholic Pressor Compounds.

Pressor compound	Formula	No. of exp.	Dose range	
	$\text{HO} \begin{array}{c} \diagup \quad \diagdown \\ \text{---} \quad \text{---} \end{array} \text{R}$ OH		Pressor cpd, $\mu\text{g/kg}$	194, $\mu\text{M/kg}$
Epinephrine HCl	$\text{R} = \begin{array}{c} \text{OH} \quad \text{NHCH}_3 \\ \quad \\ -\text{CH}-\text{CH}_2 \end{array}$	63	0.5-8.0	10-77
Norepinephrine HCl*	$\begin{array}{c} \text{OH} \quad \text{NH}_2 \\ \quad \\ -\text{CH}-\text{CH}_2 \end{array}$	23	0.5-6.0	10-77
Cobefrine HCl*	$\begin{array}{c} \text{OH} \quad \text{NH}_2 \\ \quad \\ -\text{CH}-\text{CH}-\text{CH}_3 \end{array}$	11	2.0-6.0	15-25
Epinine HCl†	$\begin{array}{c} \text{H} \quad \text{NHCH}_3 \\ \quad \\ -\text{CH}-\text{CH}_2 \end{array}$	2	6.0-16.0	15
Keprine HCl*	$\begin{array}{c} \text{O} \quad \text{NHCH}_3 \\ \quad \\ -\text{C}-\text{CH}_2 \end{array}$	2	2×10^3	15
Catechol	-H	2	$6-12 \times 10^3$	15

* Generously furnished by Dr. M. L. Tainter of the Sterling-Winthrop Research Institute.

† Generously furnished by Dr. E. J. de Beer of the Wellcome Research Institute.

were administered before and after the adrenergic compound. Thus, effects of the ordinarily pressor compounds, epinephrine HCl, norepinephrine HCl (arterenol), cobefrine HCl, and epinine HCl, were compared in the pre- and post-sympatholytic periods. Table I summarizes these experiments.

When effects of a catecholic pressor amine in the pre- and post-sympatholytic periods were examined, the post-sympatholytic effect showed two principal types of difference from the pre-sympatholytic effect:

1) Abolition or reduction in magnitude of the initial pressor spike, and 2) revelation of a depressor action, when none had been present prior to the 194; or augmentation of a depressor effect, when, in the presympatholytic period, such effect followed the primary pressor spike.

In summary. a) Magnitudes of changes 1) and 2), undergone by a given sympathomimetic compound, tended to vary in the same direction; b) Magnitudes of changes 1) and 2) sustained by the animal after equal (by weight) small doses of the different sympathomimetic amines, in the presence of a given dose of 194, showed the following order: Epinephrine > norepinephrine \approx

cobefrine c) *Duration* of post-sympatholytic pressor or depressor effects usually varied directly with the *magnitude* of such effects (except with epinephrine, as explained below.)

In most experiments with epinephrine, pressor action of doses $\leq 8 \mu\text{g/kg}$ was completely or nearly completely abolished. In every experiment with epinephrine, impressive alterations, both of types 1) and 2), were seen. Pressor-depressor effects of epinephrine were modified by 194 in a manner quite similar to that reported for Dibenamine HCl:^{6,7} nearly maximum depressor effects could be evinced with relatively small doses of epinephrine (2-6 $\mu\text{g/kg}$), and multiplying the dose by a factor as great as 10^3 produced only a prolongation of the depression, with little or no change in depth.

It is noteworthy that, in every experiment in which the 3 were compared, epinephrine consistently demonstrated substantially greater depressor activity, following the lytic agent, than did norepinephrine or cobefrine. Perceptible evidence of any depressor action was shown by small doses of norepinephrine

⁶ Nickerson, M., and Goodman, L. S., *J. Pharm. Exp. Therap.*, 1947, **89**, 167.

⁷ Biörck, G., *Acta physiol. Scand.*, 1947, **14**, 174.

and cobefrine in fewer than *half* the animals in which they were used.

Two experiments with epinine showed that alterations 1) and 2) were considerably smaller than with epinephrine. The primary pressor actions of kephrine (adrenalone) and catechol were abolished by prior infusion of 194 and were replaced by purely depressor responses. Since relatively large doses of these compounds must be used to evoke pressor effects, they were administered in the same manner as the non-catecholic pressor amines (see below). Tainter,²⁹ using ergotamine, also has achieved reversal of the pressor effect of catechol.

B. Non-catecholic pressor amines. In each experiment listed in Table II, the only injections were, in order: 1) infusion of sympatholytic compound; 2) one or two doses of 6 $\mu\text{g}/\text{kg}$ of epinephrine (a dose which invariably produces a pressor response in the *absence* of the lytic agent) to confirm the presence of a "block" of the pressor action of epinephrine; and 3) a single dose of the sympathomimetic agent being examined (the possibility of tachyphylaxis was excluded by using a single dose of any amine except epinephrine).

The effect of each pressor amine, in the presence of the stated dose of 194, is shown in Table II. The data tabulated there demonstrate that the blood pressure elevation, usually observed after the intravenous injection of large doses of any of these compounds, has been greatly reduced or abolished. Furthermore, most of these compounds are shown to exert some depressor effect.

II. Nictitating membrane. In a series of 18 experiments, doses of 6-50 $\mu\text{M}/\text{kg}$ of 194 (usually 15-20 $\mu\text{M}/\text{kg}$) were administered. Records were made of the nictitating membrane responses to faradic stimulation of the preganglionic (peripheral) stump of the homolateral cervical sympathetic trunk and to epinephrine injected into a femoral vein or into the homolateral carotid artery or both.

In general, contractions resulting from intravenous or intra-carotid epinephrine (1-8

$\mu\text{g}/\text{kg}$) were diminished considerably more, in a given animal, by a dose of 194, than were contractions of equal height resulting from standard faradic stimuli applied to the sympathetic nerve. In only 1 of 18 experiments did the 194 fail to reduce significantly the responses to epinephrine; in about one-third of these experiments, intravenous injection of 1-5 $\mu\text{g}/\text{kg}$ of epinephrine was able to evoke *no* contraction, after 194 was infused. On the other hand, response to a standard faradic stimulus was altogether unaffected by 20-25 $\mu\text{M}/\text{kg}$ of 194 in 3 of 10 experiments; and, in *no* instance, was a contraction resulting from adrenergic nerve stimulation completely prevented by 194. Moreover, sympatholysis, when present, developed much more slowly than adrenolysis. These results are similar to those reported for Dibenamine HCl.⁸

A "reversal" (*i.e.* relaxation in lieu of contraction) never occurred when epinephrine was administered after the lytic agent. Such "reversal" of epinephrine-effect was seen by Acheson (*cit.* by Morison and Lissak⁹) when he administered ergotoxine prior to the epinephrine injection. Small contractions (of nictitating membranes acutely denervated by transection of pre-ganglionic sympathetic fibres) sometimes produced by intravenous injection of doses of norepinephrine $\leq 5 \mu\text{g}/\text{kg}$, were prevented by 15 $\mu\text{M}/\text{kg}$ of 194. Similarly, larger contractions resulting from appropriate doses of propadrine or benzedrine were prevented or considerably diminished by the lytic compound. Moreover, the extremely prolonged contractions induced by suitable doses of benzedrine or desoxyephephrine could be interrupted by an infusion of 194 initiated *after* the contraction was well established.

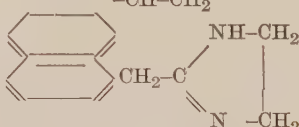
Discussion. The literature reveals reports of differences in behavior of sympathomimetic amines in the presence of different adrenolytic compounds. Table III summarizes some of these reports.

⁸ Simeone, F. A., and Sarnoff, S. J., *Surgery*, 1947, **22**, 391.

⁹ Morison, R. S., and Lissak, K., *Am. J. Physiol.*, 1938, **123**, 404.

²⁹ Tainter, M. L., *J. Pharm. Exp. Therap.*, 1930, **40**, 43.

TABLE II.
 Experiments with Non-catecholic Pressor Compounds.

Name of pressor compound	Structural formula			Dose of pressor epd, mg/kg	Dose of 194, μ M/kg	Effect after 194	
	R ₁	R ₂	R ₃			Primary \S pressor, mm Hg	Secondary depressor, mm Hg
B-phenylethyl amine HCl*	H	H	NH ₂	1.31	15	0¶	14 (18)**
			$-\text{CH}_2-\text{CH}_2-$	1.30	15	0	12 (15)
Benzedrine SO ₄	H	H	NH ₂	2.94	15	0	16 (18)
			$-\text{CH}_2-\text{CH}-\text{CH}_3$	2.94	15	0	18 (20)
Propadrine HCl*	H	H	ON NH ₂	1.80	15	0	10 (14)
			$-\text{CH}-\text{CH}-\text{CH}_3$	1.80	15	0	18 (20)
Desoxyephedrine HCl*	H	H	NHCH ₃	2.00	15	0	8 (7)
			$-\text{CH}_2-\text{CH}-\text{CH}_3$	2.00	15	6 (6)	16 (17)
				2.00	15	10 (17)	0
				1.98	15	0	?
Ephedrine SO ₄	H	H	OH NHCH ₃	1.20	15	0	6 (7)
			$-\text{CH}-\text{CH}-\text{CH}_3$	1.20	19	0	14 (10)
Vonedrine HCl*	H	H	CH ₃ NHCH ₃	2.39	25	6 (5)	32 (27)
			$-\text{CH}-\text{CH}_2-$	2.38	25	8 (19)	6 (14)
Tyramine H ₂ PO ₄	OH	H	NH ₂	0.91	15	0	14 (21)
			$-\text{CH}_2-\text{CH}_2-$	0.89	15	6 (8)	12 (15)
Paredrine HBr	OH	H	NH ₂	0.49	15	0	8 (11)
			$-\text{CH}_2-\text{CH}-\text{CH}_3$	0.49	15	0	0
Paredrinol SO ₄ *	OH	H	NHCH ₃	0.18	15	0	0
			$-\text{CH}_2-\text{CH}-\text{CH}_3$	0.36	25	6 (6)	0
Synephrine tartrate*	OH	H	OH NHCH ₃	0.74	17	0	10 (15)
			$-\text{CH}-\text{CH}_3$	0.71	14	6 (6)	0
Neosynephrine HCl*	H	OH	OH NH-CH ₃	0.03	15	8 (9)	0
			$-\text{CH}-\text{CH}_2-$	0.03	15	6 (8)	0
Privine HCl ‡				0.06	25	6 (8)	0
				0.07	15	22 (32)	0
				0.06	25	8 (8)	0
Tuamine SO ₄ †	$\text{CH}_3-(\text{CH}_2)_4-\text{CH}-\text{CH}_3$ $\quad \quad \quad $ $\quad \quad \quad \text{NH}_2$			1.90	15	6 (10)	14 (23)
				1.90	15	0	22 (26)
				1.89	25	6 (5)	20 (18)
2-Amino-6 methyl heptane SO ₄ †	$\text{CH}_3-\text{CH}-(\text{CH}_2)_3-\text{CH}-\text{CH}_3$ $\quad \quad \quad \quad \quad \quad $ $\quad \quad \quad \text{CH}_3 \quad \quad \quad \text{NH}_2$			1.00	16	0	16 (15)
				1.00	15	0	10 (9)
2-Amino-4-methyl hexane SO ₄ †	$\text{CH}_3-\text{CH}_2-\text{CH}-\text{CH}_2-\text{CH}-\text{CH}_3$ $\quad \quad \quad \quad \quad \quad $ $\quad \quad \quad \text{CH}_3 \quad \quad \quad \text{NH}_2$			1.78	15	0	0
				1.83	15	0	8 (11)

* Generously furnished by the Smith, Kline, and French Laboratories.

† Generously furnished by Dr. K. K. Chen of the Lilly Research Laboratory.

‡ Generously furnished by Ciba Pharmaceutical Products, Inc.

§ Refers to initial pressor response which ordinarily follows intravenous injection of sympathomimetic compound.

|| Refers to depressor effect following the diminished initial pressor response, or to the initial depressor effect when primary pressor effect has been abolished.

¶ "0" refers to a B.P. response ≤ 4 mm Hg.

** Each transverse row of data is extracted from 1 experiment.

Quantities in parentheses are percentage changes, of pre-injection blood pressure, corresponding to absolute changes in mm Hg.

TABLE III.
Effects of Several Chemical Species of Adrenolytic Agents on Sympathomimetic Compounds.

Sympathomimetic pressor compound	Adrenolytic agent				194*
	Ergot alkaloids	Dioxane derivatives (Fourneau)	Prisco- (benzyl- imidazoline)	N-Mustard derivatives	
Catechol	R ²⁹				R
Epinephrine	R ²⁹	R ¹⁵			PR
Norepinephrine	B†			R ³	PR
Cobefrine	B†			R ³	PR
Kephrine	R ²⁹	R ¹⁵		R ³	R
B-phenylethylamine		B ¹⁵			R
Benzedrine	B ^{7,12}	B ¹⁵	B ¹⁶	R ^{3,7}	R
Propadrine			B ¹⁶	R ³	R
Desoxyephedrine	NR ²⁷				R
Ephedrine	B†			O ¹⁷	
	R ^{10,11,12}	B ¹⁵	B ¹⁶	R ³	R
Vonedrine			B ¹⁶		R
Tyramine	R ²⁸	B ¹⁵	R ¹⁶	R ³	R
Paredrine			B ¹⁶	R ³	R
Paredrinol				R ³	R
Synephrine	B ¹³		B ¹⁶		R
Neosynephrine	R ⁵	R ¹⁵	B ¹⁶	R ³	B
Privine			B ¹⁶	R ³	B
Aliphatic pressor compounds	O ¹⁴		R ¹⁶		R

O—No effect reported.

B—Partial or complete abolition of pressor effect; depressor activity not increased.

PR—Partial reversals; reduction or abolition of pressor effects consistently achieved; secondary depressor action sometimes produced or augmented.

NR—No reversals reported.

R—Complete or nearly complete reversals in some experiments.

* Experiments cited in present report.

† Controversy persists regarding reversal of ephedrine effect.

‡ In 2 experiments on cats, we found that 5 mg/kg of dihydroergocristine methane sulphonate and dihydroergokryptine methane sulphonate, respectively, partially or completely blocked pressor effects, but revealed no significant depressor action.

Numbers correspond to references in bibliography.

It is noteworthy that each of the pressor substances listed has demonstrated significant *depressor* activity under the influence

of at least one lytic compound. It is likely that such depressor effects could be elicited in some instances (including our own experiments) in which only "blocking" of pressor properties has been reported, if modifications of experimental conditions, dosage, etc., were introduced.

In this connection, the influence of species of experimental animal is remarkable. For example, it has been shown that a variety of lytic agents (including ergot alkaloids,¹⁸ dioxane derivatives,¹⁵ and N-mustards⁷ can reverse epinephrine-hypertension in

¹⁰ Barger, G., *Ergot and Ergotism*, London, 1931.

¹¹ Goodman, L. S., and Gilman, A., *The Pharmacological Basis of Therapeutics*, New York, 1941.

¹² Sollman, T., *A Manual of Pharmacology*, Philadelphia, 1947.

¹³ Tainter, M. L., and Seidenfeld, M. A., *J. Pharm. Exp. Therap.*, 1930, **40**, 23.

¹⁴ Ahlquist, R. P., *J. Pharm. Exp. Therap.*, 1945, **85**, 283.

¹⁵ Bovet, D., *J. Suisse Méd.*, 1943, **73**, 153.

¹⁶ Ahlquist, R. P., et al., *J. Pharm. Exp. Therap.*, 1947, **89**, 271.

¹⁷ De Vleeschouwer, G. R., *PROC. SOC. EXP. BIOL. AND MED.*, 1947, **66**, 151.

¹⁸ Dale, H. H., *J. Physiol.*, 1906, **34**, 163.

²⁷ Hauschild, F. (cit. by Haley, T. J., *J. Am. Pharm. Assn.*, 1947, **36**, 161).

²⁸ Tainter, M. L., *J. Pharm. Exp. Therap.*, 1926-27, **30**, 163.

the cat but can only *prevent* it in the rabbit. This striking concordance of results may reflect a basic physiologic difference which can be explored for information related to the anatomical and biochemical foundations of sympatholytic activity and of the depressor effects of sympathomimetic substances.

Having found that paredrine reverses the pressor effect of vonedrine in the dog whereas priscol simply blocks it, Ahlquist¹⁹ postulated that the former phenomenon represented an "actual reversal of action and not an unmasking of a normally present response." This interpretation hardly seems necessary, since we have demonstrated that the effects of vonedrine readily can be reversed by 194.

Hunt²³ has found that certain bis- β -chloroethyl derivatives of the N-mustard series block the blood-pressure changing effects of both *pressor* and *depressor* amines. If confirmed, this phenomenon would be unique since other compounds which have been shown to prevent certain inhibitory effects of epinephrine are unable to block the blood-pressure reducing action of epinephrine.^{15,26}

Drug 194 is closely related chemically to Dibenamine HCl (dibenzyl- β -chloroethylamine HCl) and somewhat more remotely related to other N-mustard blocking agents concerning which publications have appeared recently.^{3,20,21,22,23} Its effects, in our hands, were comparable to those of Dibenamine. However, the latter compound, as well as 194, usually caused a slow progressive fall in blood-pressure, sometimes to shock levels. Usually the fall in blood pressure terminated at a level of 60-100 mm of Hg. Fifteen μ M/kg of 194 caused reversal of epinephrine (6 μ g/kg) for at least 2-6 hours; whereas Biorck,⁷ using comparable doses of epinephrine and Dibenamine which he injected rapidly

(during 1-2 min.) found little or no adrenolysis after one hour.

The experiments reported here do not appear to contribute measurably to answering the problem of relating sympathomimetic chemical constitution, or vascular sites of action (where these are known), or both, to the capacity of a lytic substance to annul or reverse the effects of such sympathomimetic amines. The comprehensive ability of 194 to prevent certain pharmacodynamic effects of these sympathomimetic compounds does seem to support the concept of their eventual action, direct or indirect, on a point or points which are rendered inactive or inaccessible, by reason of previous irreversible, or only slowly reversible, reaction with 194. ("Indirect" action of mimetic molecules is exemplified by the postulate of Gaddum and Kwiatowski²⁴ regarding the mode of action of ephedrine.) The work of Bergmann *et al.*²⁵ supports the possibility that 194 (and other N-mustard blocking agents) might affect more than one reactive "receptor" group.

Summary and Conclusions. 1. Drug 194 (N-benzyl-N- β -phenylisopropyl- β chloroethylamine hydrochloride), has been shown to be a potent antagonist of certain excitatory effects of a number of sympathomimetic agents. In the cat, 194 reduced or abolished pressor responses to aliphatic and aromatic amines and to catechol. In the presence of 194, all the compounds, except neosynephrine, privityne, and paredrinol, manifested depressor activity.

2. The effects of nerve stimulation on the nictitating membrane of the cat were less effectively blocked by 194 than were the effects of epinephrine. 194 also could prevent or interrupt nictitating membrane contractions induced by sympathomimetic amines other than epinephrine.

¹⁹ Ahlquist, R. P., *J. Am. Pharm. Assn.*, 1946, **35**, 348.

²⁰ Achenbach, P., and Loew, E. R., *Fed. Proc.*, 1947, **6**, 304.

²¹ Loew, E. R., and Micetich, A., *Fed. Proc.*, 1947, **6**, 351.

²² Loew, E. R., Micetich, A., and Achenbach, P., *Fed. Proc.*, 1947, **6**, 351.

²³ Hunt, C. C., *Fed. Proc.*, 1948, **7**, 229.

²⁴ Gaddum, J. H., and Kwiatowski, L., *J. Physiol.*, 1938, **94**, 87.

²⁵ Fruton, J. S., Stein, W. H., and Bergmann, M., *J. Org. Chem.*, 1946, **11**, 559.

²⁶ Rothlin, E., *Bull. Acad. Suisse Sci. Med.*, 1946-47, **2**, 1.

A Method for Demonstrating Antibodies in Rabbit Sera Against Histoplasmin by the Collodion Agglutination Technic.

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Serologic tests for histoplasmosis may prove to be valuable adjuncts for both the diagnosis of the disease and for a more critical evaluation of the significance of the positive skin test reaction to histoplasmin. To date, the complement fixation tests reported from this laboratory¹ and others^{2,3} have been the only *in vitro* immunologic procedures described. An improved method for the preparation and use of sensitized collodion particles for the detection of specific antibodies in blood sera has been reported recently by Cavelti.⁴ The collodion agglutination test appeared to offer a potential technic for measuring antibodies against histoplasmin in positive skin reactors and in experimental sera derived from animals immunized with *Histoplasma capsulatum*. This report describes the results obtained in testing the blood sera of normal and immunized rabbits against suspensions of collodion particles sensitized with histoplasmin.

Methods. The collodion particles used in these studies were prepared by the method of Cavelti.⁴ A suspension of particles diluted to match the turbidity of the McFarland scale No. 2 was sensitized by adding an equal volume of stock histoplasmin H-40* diluted

1:200 in physiologic saline. This optimal dilution of histoplasmin was determined by titration as shown in Table I. The histoplasmin and collodion mixture was shaken thoroughly and kept at room temperature for one hour to permit sensitization of the collodion particles. All serum specimens were inactivated at 60°C for 30 minutes and serial dilutions made in physiologic saline solution so that each tube contained 0.5 ml. To each serum dilution 0.5 ml of the suspension of sensitized particles was added. The tubes were shaken vigorously, incubated at room temperature for 2 hours and then centrifuged for 3 minutes at 1400 rpm in a No. 2 International horizontal centrifuge. The results were read by gently flicking the tubes in front of a black screen above which a microscope lamp served as a source of light. Agglutinations were considered 4+ when the diameter of the flakes was about 1 to 1½ mm or over with the decreasing gradations of 3+, 2+ and 1+. In the 1+ reaction the particles were very fine but still readily visible. A test was considered doubtful (±) if particles were just barely visible and negative when no particulate matter was observed after resuspension.

Young healthy rabbits were immunized with yeast phase strains of *H. capsulatum* and with the other agents used in this study according to the method described in a previous report.¹ The sera of rabbits immunized with 3 strains of *H. capsulatum* (G-2, G-5, G-6),† 2 strains of *Blastomyces dermatitidis*

† The G-2, G-5 and G-6 yeast phase strains of *H. capsulatum* were reverted from the mycelial phase of strains 715, 952 and 650, respectively, from the collection of Dr. N. F. Conant at Duke University. The A-1 strain of *B. dermatitidis* and A-5 (Duke 930) and the strain of *B. brasiliensis* (Duke 871B) are all human isolates received from Dr. Conant.

¹ Saslaw, S., and Campbell, C. C., *J. Lab. and Clin. Med.*, 1948, **33**, 811.

² Salvin, S. B., *Proc. Soc. Exp. Biol. and Med.*, 1947, **66**, 342.

³ Tenenbergh, D. J., and Howell, A., *Pub. Health Rep.*, 1948, **63**, 163.

⁴ Cavelti, P. A., *J. Immunol.*, 1947, **57**, 141.

* Issued by the Office of Field Studies, Tuberculosis Control Division, United States Public Health Service. Lot H-40 histoplasmin was prepared from the pooled broth filtrates of the mycelial phase of 3 strains of *Histoplasma capsulatum* and standardized by the method outlined by Howell.⁵

⁵ Howell, A., *Pub. Health Rep.*, 1947, **62**, 631.

TABLE I.
Determination of the Optimal Dilution of Histoplasmin for Collodion Agglutinations Using Serum Prepared in Rabbits Against the G-2 Strain of *H. capsulatum*.

Dil. of histoplasmin before mixture with equal quantity of collodion*	Positive serum dilutions 0.5 ml							Controls†	
	0.5 ml	1:5	1:10	1:20	1:40	1:80	1:160	1:320	Antigen Serum
1:5	—	—	—	—	—	—	—	—	—
1:10	—	—	—	—	—	—	—	—	—
1:20	—	—	—	—	—	—	—	—	—
1:40	2+	—	—	—	—	—	—	—	—
1:100	4+	4+	3+	1+	—	—	—	—	—
1:200†	4+	4+	3+	3+	1+	±	—	—	—
1:400	3+	2+	1+	±	—	—	—	—	—
1:800	2+	1+	1+	±	—	—	—	—	—
1:1000	±	±	±	±	—	—	—	—	—
1:2000	—	—	—	—	—	—	—	—	—
1:4000	—	—	—	—	—	—	—	—	—
1:8000	—	—	—	—	—	—	—	—	—

* Collodion diluted to equal turbidity of No. 2 McFarland nephelometer.

† 1:200 dilution selected as optimum dilution on basis of comparative titers.

‡ In antigen control sensitized particles were mixed with 0.5 ml of saline. In serum control positive serum (1:5 and 1:10) was mixed with unsensitized collodion. Normal rabbit serum was similarly titrated as above and was negative throughout.

TABLE II.
Results of Collodion Agglutination Tests with Rabbit Sera.

Sera	Animal No.	Serum dilutions—0.5 ml					
		1:5	1:10	1:20	1:40	1:80	1:160
G-2 (<i>H. capsulatum</i>)	7	3+	2+	1+	—	—	—
G-2 " "	8	4+	4+	2+	2+	—	—
G-5 " "	10	4+	4+	2+	2+	1+	—
G-5 " "	11	2+	2+	1+	±	—	—
G-6 " "	12	2+	2+	1+	±	—	—
A-1 (<i>B. dermatitidis</i>)	14	+	—	—	—	—	—
A-5 " "	15	+	+	—	—	—	—
<i>B. brasiliensis</i>	16	±	—	—	—	—	—
<i>S. schenckii</i>	19	±	—	—	—	—	—
<i>C. albicans</i>	21	±	—	—	—	—	—
<i>C. neoformans</i>	26	—	—	—	—	—	—
*Normal rabbit sera (20)	31-50	—	—	—	—	—	—

* All of 20 normal rabbit serum specimens failed to show any agglutination. Duplicate control titrations using unsensitized collodion particles diluted with an equal quantity of saline showed no agglutinations with any of the above sera.

TABLE III.
Effect of Varying Incubation Temperature on Collodion Agglutination.

Sera	2 hr incubation at °C	Serum dilutions—0.5 ml				
		1:5	1:10	1:20	1:40	1:80
G-2	Room temp.	3+	2+	1+	—	—
Normal	" "	—	—	—	—	—
G-2	37	1+	±	—	—	—
Normal	37	—	—	—	—	—
G-2	52	—	—	—	—	—
Normal	52	—	—	—	—	—

(A-1 and A-5)* and one each of *Blastomyces braziliensis*,* *Candida albicans*,† *Cryptococcus neoformans*† and *Sporotrichum schenckii*† were tested against collodion particles sensitized with histoplasmin. In addition, sera of normal stock rabbits were similarly investigated.

Results. The data as presented in Table II reveal that the sensitized collodion particles were uniformly agglutinated in the presence of sera from animals immunized with all 3 strains of *H. capsulatum* with serum titers ranging from 1:20 to 1:80. None of the other fungal antisera tested gave positive agglutinations except *B. dermatitidis* antisera, which reacted in low titer. Twenty specimens of sera from normal stock rabbits likewise failed to agglutinate the sensitized particles. In control studies collodion particles diluted with saline instead of histoplasmin showed no agglutinations in the presence of the same series of serum specimens.

Effects of varying incubation time and temperature. In order to ascertain the conditions for optimum incubation, 2 sets of experiments were conducted. In one study known positive serum and sensitized collodion mixtures were incubated at 37°C, 52°C, and at room temperature for 2 hours. As can be seen in Table III the agglutination reactions were stronger after incubation at room temperature than at 37° C while at 52° C no agglutination occurred. The normal serum control was negative throughout. In the second series of studies the tubes were placed in the refrigerator overnight after the routine 2 hour room temperature incubation and reading. The following day the tubes were recentrifuged in the usual manner and read. Unlike the results following primary incubation, many instances of non-specific agglutinations occurred with normal and heterologous sera while the specific sera gave higher "titers" (Table IV). Thus to obtain optimum and specific results the 2-hour incubation at room temperature was employed as the method of choice.

Summary. A method for utilizing sus-

TABLE IV.

Specificity of Agglutination Results after 2-Hour Incubation at Room Temperature as Compared to Overnight Incubation in Refrigerator Using Collodion Sensitized with Histoplasmin.

Sera	Rabbit No.	Serum dilutions											
		Room temperature						Refrigerator					
		1:5	1:10	1:20	1:40	1:80	1:160	1:5	1:10	1:20	1:40	1:80	1:160
G-2 (<i>H. capsulatum</i>)	7	3+	3+	1+	—	—	—	4+	4+	3+	1+	1+	—
G-5 " "	10	4+	4+	2+	1+	—	—	4+	4+	4+	3+	2+	+
G-6 " "	12	2+	2+	1+	—	—	—	3+	3+	3+	1+	—	—
<i>B. braziliensis</i>	16	+	+	—	—	—	—	3+	2+	3+	—	—	—
A-5 (<i>B. dermatitidis</i>)	15	+	+	—	—	—	—	3+	1+	1+	+	—	—
<i>C. albicans</i>	21	+	—	—	—	—	—	1+	1+	—	—	—	—
<i>C. neoformans</i>	26	+	—	—	—	—	—	1+	1+	—	—	—	—
Normal	31	—	—	—	—	—	—	+	1+	—	—	—	—
" "	32	—	—	—	—	—	—	2+	+	—	—	—	—

† Isolated from patient material at the Army Medical Center.

pensions of collodion particles sensitized with histoplasmin for the determination of antibodies against histoplasmin is described. The sensitized suspensions uniformly showed the presence of antibodies against histoplasmin in rabbits which had been immunized with 3 different strains of *H. capsulatum*. Antisera against *B. dermatitidis* reacted in low

dilution, while that of *B. braziliensis*, *S. schenckii*, *C. neoformans* and *C. albicans* as well as normal rabbit sera failed to agglutinate the histoplasmin-coated particles. These results suggest the use of this technic with human sera. The findings of such studies are now being analyzed in this laboratory.

16552

Intestinal Absorption of Vitamin A from Aqueous and Oily Menstruum.*

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Oral intake of vitamin A in aqueous dispersions produces a significantly higher rise of the blood vitamin A level than in oily menstruum.^{1,2,3} This was considered evidence of improved intestinal absorption, especially since after intake of the aqueous preparation, the fecal excretion of vitamin A appeared reduced² and the storage in the liver increased.^{2,4} The use of aqueous preparations appeared especially superior in improving the response to vitamin A in patients with liver⁵ or celiac⁶ disease. This suggested the direct observations of the intestinal absorption of vitamin A in aqueous and oily solution in rats by fluorescence microscopy.⁷

Material and Method. Thirty-three albino rats, all weighing between 140 and 170 g and previously on a stock diet, were sacrificed at different intervals following feeding of various doses of vitamin A as unsaponifiable fraction of fish liver oil in corn oil, or dispersed in water with a sorbitan monolaurate derivative. Both preparations[†] contained 25,000 U.S.P. units of vitamin A per cc and were made from the same vitamin A concentrate. Pieces of duodenum, upper jejunum, lower jejunum and ileum were fixed in 10% formalin. Frozen sections were prepared and studied microscopically for vitamin A fluorescence.⁸ Total amount of vitamin A fluorescence and its site were recorded. Vitamin A fluorescence is seen in the intestinal wall of the rat only after feeding high doses of vitamin A.⁷

Results. Independent of variations of the amount of vitamin A fed and of the time interval between administration and sacrifice (Table I), the amount of vitamin A in the intestinal wall was larger after an equal dose of vitamin A in aqueous than in oily menstruum. The difference was especially evident with reduction of the amount of vita-

* Supported by a grant from Endo Products, Inc., Richmond Hill, N. Y.

¹ Kramer, B., Sobel, A. E., and Gottfried, S. P., *Am. J. Dis. Child.*, 1947, **73**, 543.

² Lewis, J. M., Bodansky, O., Birmingham, J., and Cohan, S. Q., *J. Pediat.*, 1947, **31**, 496.

³ Popper, H., Steigmann, F., and Dyniewicz, H. A., *J. Lab. and Clin. Med.*, 1947, **32**, 1403.

⁴ Sobel, A. E., Sherman, M., Lichtblau, O., Snow, S., and Kramer, B., *J. Nutrition*, 1948, **35**, 225.

⁵ Popper, H., Steigmann, F., and Dyniewicz, H. A., *Gastroenterology*, in press.

⁶ May, C. D., and Lower, C. U., *J. Clin. Invest.*, 1948, **27**, 226.

⁷ Popper, H., and Volk, B. W., *Arch. Path.*, 1944, **38**, 71.

[†] Supplied by Dr. Samuel M. Gordon of Endo Products Inc.

⁸ Popper, H., *Phys. Rev.*, 1944, **24**, 205.

⁹ Frazer, A. C., *Phys. Rev.*, 1946, **26**, 103.

TABLE I.

Distribution of Vitamin A in Intestinal Wall of Rats Killed at Different Intervals After Feeding of Various Amounts of Vitamin A in Oily and Aqueous Menstruum.

Rats examined	Units of vitamin A given, $\times 1000$	Time before rats were killed, hr	Site of fluorescence peak in jejunum		Amt of fluorescence at peak	
			Oily*	Aqueous*	Oily*	Aqueous*
6	5	1	U† or Lt†	U	\pm to +	++-+++
2	15	1	L	—	+++	—
8	25	1	L	U	+++	++++
4	25	24	L	U	+	++-+++
4	25	48	L	U	0- \pm	++
5	25	72	L	U	0- \pm	++
2	75	1	U	—	++++	—
1	75	48	L	—	++	—
1	75	72	U and L	—	++	—

* Menstruum. † L—lower; U—upper.

min A fed or extension of the time interval. In such instances, hardly anything was seen after administration of the oily solution, whereas, the animals fed the aqueous dispersion still revealed significant amounts. One hour after administration of 25,000 units of vitamin A the peak of absorption occurred usually higher in the upper jejunum with the aqueous preparation while the lower jejunum showed the most after feeding vitamin A in oil;⁷ vitamin A fluorescence was found after feeding the aqueous preparation in the lacteals and histiocytes of the villi at all examined levels, whereas, it was found **only** in the villi of the jejunum after the same amount in oil. In the lacteals of muscularis and subserosa it was found only in the jejunum, however, in much higher degree after administration of the aqueous dispersion. Three days after feeding of 25,000 units of vitamin A in aqueous preparation, considerable vitamin A fluorescence was encountered in the lamina propria of the villi and the lacteals of muscularis and subserosa, whereas the epithelial lining was free of it. Administration of the same amount of vitamin A in oil failed to produce significant fluorescence in the same time interval, whereas, 75,000 units in oil produced a similar picture as 25,000 units in aqueous emulsion. In general, the administration of vitamin A in oil produced approximately the same picture as feeding in aqueous dispersion if a triple amount in oil was given. No difference was found in location and degree of the vitamin A fluorescence of the intestinal con-

tent which decreased rapidly from the jejunum downward.

Discussion. Direct observation confirmed the indirect evidence obtained by the tolerance curve that vitamin A is absorbed in increased amounts if fed in aqueous instead of in oily menstruum. Rough estimation suggests that three times as much vitamin A is absorbed if given dispersed in water in agreement with findings on liver storage tests.⁴ The peak of the absorption occurs in higher parts of the jejunum and vitamin A migrates quicker through the intestinal wall and is retained longer if given in aqueous dispersion. However, these differences seem to be only expressions of larger amounts of vitamin A in the intestinal wall since tripling the dose of the oily solution produces the identical histologic picture. No evidence was seen that vitamin A is absorbed or transported in a qualitatively different manner if given in water. Absorption of vitamin A through blood vessels is not visible as could have been expected after the studies of Frazer⁹ and be explained by increased lipolysis. Fine particulate state of vitamin A, therefore, hastens its passage through the epithelium of the intestinal villi, but does not change the mode of its absorption within the villi. This supports the opinion that the faulty absorption of vitamin A in oil, which is found in hepatic or celiac disease, is due to a defect in emulsification and not in lipolysis.

The delayed disappearance of vitamin A after administration of large amounts indi-

cates that vitamin A is temporarily stored in the lamina propria of the villi. This lag in release after excessive absorption requires further explanation.

The increased absorption of vitamin A in aqueous dispersion is of practical significance because of sparing of vitamin A, the correction of absorption difficulties^{4,5} and the increased speed of absorption. The latter was claimed to be of importance in the treatment of infants with tendency to vomiting.¹

Summary. Observation of the vitamin A fluorescence in the intestine of rats showed

that greater amounts of vitamin A—approximately three times as much—were absorbed if the latter was administered in an aqueous instead of an oily menstruum. In addition vitamin A in water penetrated the intestinal wall faster, the peak of absorption occurred at a higher level of the intestine and vitamin A remained longer in the villi. These differences were explained by passage of increased amounts of vitamin A through the epithelium if given in aqueous dispersion and not by a different physical state of vitamin A after passage.

16553

Amino Acid Imbalance of Zein: Relation to Nicotinic Acid Deficiency in the Chick.*

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Krehl *et al.*¹ were the first to show that a nicotinic acid deficiency could be induced in the rat by feeding corn as a part of a low-protein diet. Subsequently² it was observed that *l*-tryptophane as well as nicotinic

acid was effective in counteracting the growth depression in rats caused by the feeding of corn. The activity of tryptophane in this connection indicates that it is an important precursor of nicotinic acid.^{3,4,5} Tryptophane-low proteins such as gelatin, zein and acid-hydrolyzed casein have been shown to behave similarly to corn.^{6,7} That the action of these proteins may be due to amino acid imbalances has been suggested in work with the rat^{8,9} and the chick.^{10,11} The subject

* Scientific Paper No. A201. Contribution No. 2117 of the Maryland Agricultural Experiment Station (Department of Poultry Husbandry).

A preliminary report of part of this work was published in *Fed. Proc.*, 1947, **6**, 257.

We are indebted to Merck & Co., Inc., Rahway, N. J., for crystalline vitamins; Wilson & Co., Inc., Chicago, Ill., for 2X gelatin; Allied Mills, Inc., Portsmouth, Va., for soybean oil; Abbott Laboratories, North Chicago, Ill., for Haliver oil; U. S. Industrial Chemicals, Inc., Baltimore, Md., for DL-methionine; and to Lederle Laboratories, Pearl River, New York, for pteroylglutamic acid.

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¹ Krehl, W. A., Teply, L. J., and Elvehjem, C. A., *Science*, 1945, **101**, 283.

² Krehl, W. A., Teply, L. J., Sarma, P. S., and Elvehjem, C. A., *Science*, 1945, **101**, 489.

³ Rosen, F., Huff, J. W., and Perlzweig, W. A., *J. Biol. Chem.*, 1946, **163**, 343.

⁴ Singal, S. A., Briggs, A. P., Sydenstricker, V. P., and Littlejohn, J. M., *J. Biol. Chem.*, 1946, **166**, 573.

⁵ Sarett, H. P., and Goldsmith, G. A., *J. Biol. Chem.*, 1947, **167**, 293.

⁶ Krehl, W. A., Sarma, P. S., and Elvehjem, C. A., *J. Biol. Chem.*, 1946, **162**, 403.

⁷ Briggs, G. M., *J. Biol. Chem.*, 1945, **161**, 749.

⁸ Krehl, W. A., Henderson, L. M., de la Huerga, J., and Elvehjem, C. A., *J. Biol. Chem.*, 1946, **166**, 531, and Henderson, L. M., Deodhar, T., Krehl, W. A., and Elvehjem, C. A., *J. Biol. Chem.*, 1947, **170**, 261.

of imbalances in nutrition in general has recently been reviewed.¹²

Reports from this laboratory^{7,10} have pointed out the growth inhibiting activities of corn, gelatin, and zein when fed to chicks receiving diets low in nicotinic acid. By employing an amino acid mixture simulating gelatin, it was found¹¹ that the growth depression caused by this protein was due largely to the combined action of glycine, arginine, and alanine emphasizing the role played by amino acid imbalance. The feeding of nicotinic acid completely reversed the actions of gelatin^{7,10,11} and the amino acids.¹¹

The present report concerning the corn protein, zein, is a study similar to that carried out with gelatin. Since zein contains no glycine and very little arginine, these amino acids obviously were not primarily concerned with the growth depression obtained by the feeding of this protein. Other factors were apparently involved. Moreover, it seemed desirable to investigate the role of amino-acid imbalance of zein to determine the extent to which growth depression could be explained on this basis or if a part could be attributed to contamination of the protein with a "pellagragenic" agent reported¹³ to exist in corn.

Experimental. Day-old New Hampshire chicks of mixed sexes were used. The chicks were distributed according to weight, confined in electrically heated wooden cages with wire floors and given feed and water *ad libitum*. Weighings and other observations were made weekly. All substitutions in the basal diet 113GN were made at the expense of cerelose (Table I).

Results. Table II summarizes the re-

TABLE I.
Composition of Basal Diet 113GN.

	%
Cerelose	71.4
Casein (crude)	18.0
Mineral mixture 1M	6.0
Soybean oil	4.0
<i>dl</i> -Methionine	0.3
Choline chloride	0.2
<i>i</i> -Inositol	0.1
Vitamins (mg/100 g)	
Thiamine HCl	0.4
Riboflavin	0.8
Ca pantothenate	2.0
Pyridoxine HCl	0.6
Biotin	0.02
Para-aminobenzoic acid	0.2
2-Methyl-1,4-naphthoquinone	0.1
Alpha-tocopherol	0.5
Pteroylglutamic acid	0.2
Vitamins A and D ₃ , 1200 U.S.P. and 170 A. O. A. C. units, respectively, by dropper weekly	

sults obtained by feeding the corn protein, zein (commercial preparation) and an amino-acid mixture simulating zein to chicks. The addition of 15% of zein to the diet decreased growth slightly and increased the incidence of blacktongue (compare groups 1 and 5). Growth was depressed somewhat more with 5% additional gelatin (compare Groups 1 and 2). However, the feeding of the amino-acid mixture produced much more severe results than did zein (compare Groups 5 and 6). Less severe results were obtained with the modified amino-acid mixture (compare Groups 6 and 7), but the severity of its action was still greater than zein (compare Groups 5 and 7). Thus it appears that the growth depression caused by the feeding of zein can be explained entirely on the basis of its amino-acid constituents, which likewise is true for gelatin. The growth-depression effect of zein could be largely duplicated by a mixture of 5 amino acids—glutamic acid, leucine, alanine, proline, and phenylalanine. These are the predominant amino acids of this protein. The residual amino acids were additive in producing a further growth-depressing action. It is evident that the unnatural (D) forms of the amino acids, which were used in several instances, were not greatly responsible in themselves for the effects observed (unpublished data). The

⁹ Singal, S. A., Sydenstricker, V. P., and Littlejohn, J. M., *J. Biol. Chem.*, 1947, **171**, 203.

¹⁰ Briggs, G. M., Groeschke, A. C., and Lillie, R. J., *J. Nutrition*, 1946, **32**, 659.

¹¹ Groeschke, A. C., and Briggs, G. M., *J. Biol. Chem.*, 1946, **165**, 739.

¹² Elvehjem, C. A., and Krehl, W. A., *J. Am. Med. Assn.*, 1947, **135**, 279.

¹³ Woolley, D. W., *J. Biol. Chem.*, 1946, **163**, 773.

TABLE II.

Group No.	Supplement to basal diet 113GN	No. chicks	No. died	Avg wt 4 weeks, g	No. black-tongue
1	5% gelatin	6	0	175	2
2	10% "	6	2	133	5
3	As 1 + 10 mg % nicotinic acid	6	0	235	0
4	As 2 + 10 " % " " "	6	0	244	0
5	As 1 + 15% zein	6	1	155	5
6	As 1 + 15% amino-acid mixture*	6	5	61	6
7	As 1 + 11.5% modified amino-acid mixture†	6	1	110	5
8	As 5 + 10 mg % nicotinic acid	6	0	221	0
9	As 6 + 10 " % " " "	6	0	226	0

* *L*-Glutamic acid 3.90, *L*-leucine 3.60, *D*L-alanine 1.55, *L*-proline 1.45, *D*L-phenylalanine 1.00, *L*-tyrosine 0.85, *D*-isoleucine 0.65, *D*L-aspartic acid 0.50, *D*L-methionine 0.35, *D*L-threonine 0.35, *D*L-valine 0.35, *D*L-serine 0.15, *L*-histidine 0.15, *L*-arginine 0.15.

† This mixture contained glutamic acid, leucine, alanine, proline, and phenylalanine at the same levels as used in the above amino-acid mixture.

reason that zein was less depressing on growth than the amino-acid mixture was probably due to the presence of small amounts of naturally occurring tryptophane¹⁴ and/or nicotinic acid in zein. The most significant point to observe, however, is the counteraction of the effects of zein and of the amino-acid mixture with low levels of nicotinic acid (compare Groups 5, 6, 8, and 9). Furthermore, these data demonstrate that nicotinic acid is involved in some manner in the metabolism of amino acids.

Since more than maximal growth depression was obtained with the amino-acid mixture simulating the protein zein, it is logical to assume that the commercially prepared zein

was not contaminated with a "pellagrigenic" agent. Furthermore, it is safe to state that a large part of the inhibitory action of corn in chick pellagra may be attributed to its predominant protein, zein, and that the presence of a special "pellagrigenic" agent reported to exist in corn plays a relatively minor part, if any.

Summary. By use of an amino-acid mixture simulating zein, it was found that chick-pellagra symptoms in chicks caused by the feeding of zein were due to the cumulative action of the amino-acid constituents of this protein. Glutamic acid, leucine, alanine, proline, and phenylalanine, the amino acids occurring in greatest amounts, seem to be principally involved in this connection.

¹⁴ Block, R. J., and Mitchell, H. H., *Nutr. Abst. and Rev.*, 1946-47, **16**, 249.

16554

Inhibition of Testicular Hyaluronidase Activity by Rutin.*

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The term vitamin P was introduced by Armentano, Szent-Gyorgyi, and co-workers¹

to designate an essential substance other than ascorbic acid, regulating capillary permeability and fragility. In the guinea pig²

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¹ Armentano, L., Bentsah, A., Beres, T., Rusnyak, S., and Szent-Gyorgyi, A., *Deut. Med. Wochsch.*, 1936, **62**, 1325.

and in the rat,³ vitamin P deficiency produces a decreased capillary resistance. Scarborough⁴ has shown that in the human, lack of vitamin P leads to spontaneous petechial hemorrhages, lassitude and rheumatic pains. The condition rapidly responds to vitamin P, while ascorbic acid is without effect.

Parrot and Lavollay⁵ have suggested that the vitamin acts indirectly by inhibiting the oxidation of some product of adrenalin metabolism. This as yet unidentified compound is said to maintain normal capillary resistance. This action is supposedly antagonized by histamine which decreases capillary resistance. The normal capillary state is believed to be a balance between these two factors. Chambers and Zweifach⁶ have cast doubt on the theory that histamine normally influences capillary permeability. Injections of histamine locally or intravenously, in doses sufficient to produce arteriolar dilatation, fail to increase capillary permeability. Only when the concentration of histamine is sufficient to produce visible endothelial damage, does increased capillary permeability occur.

There is evidence to suggest that hyaluronidase might replace histamine in the schema of Parrot and Lavollay. Duran-Reynals⁷ has recently reviewed the experimental findings which indicate that hyaluronidase increases capillary permeability. He believes that small amounts of hyaluronidase are normally present in most, if not all tissues. Wislocki, Bunting and Dempsey⁸ have shown that arteries, arterioles and veins contain a metachromatic ground substance, probably chondroitin sulfate. Meyer and Ragan⁹

have suggested that this substance which is subject to attack by hyaluronidase, may be a constituent of capillary walls. In view of the foregoing, the possibility suggested itself that widely distributed hyaluronidase normally maintains tissue and capillary permeability and its overfunction is prevented by vitamin P. Thus a deficiency in vitamin P would lead to an excessive hyaluronidase activity which would weaken all ground substances including that present in capillary walls, and consequently would increase tissue as well as capillary permeability. With this hypothesis in mind, it was decided to test the possible antihyaluronidase activity of rutin, a vitamin P-like flavonol derivative.

Procedure. Forty-four albino rats were used, weighing 250-400 g. Ten animals were given rutin[†] intraperitoneally 3.5 hours before the hyaluronidase. The dose was 200 mg dissolved in 1 cc propylene glycol. Ten animals were similarly given 1 cc propylene glycol, but no rutin. Fourteen animals were not pretreated. The hyaluronidase was prepared in a 2% solution in 0.9% saline. The dry powder assayed 75 T.R.U. per mg and was purchased from the Tremond Company, New York.

The freshly prepared enzyme solution was gently mixed with an equal volume of doubly filtered Higgins india ink and 0.1 cc of the final solution was injected intradermally into the right flank of all animals. Into the left flank 0.1 cc of a solution of equal volumes of ink and saline was injected intradermally. Twenty-two hours later, the rats were killed with ether and skinned. The margins of the black areas on the inner surface of the skins were traced out and transposed onto cellophane paper. The enclosed areas were then measured with a planimeter. The results are presented in tabular form and analyzed statistically.

Summary. From the data it can be seen that rutin inhibits the spread of intradermally injected india ink to which hyaluronidase or saline is added. The effect is statistically highly significant. From the analysis of

² Zacho, C. E., *Acta. Path. Microbiol. Scand.*, 1939, **16**, 144.

³ Rusznayak, S., and Benko, A., *Science*, 1941, **25**, 94.

⁴ Scarborough, H., *Lancet*, 1940, **239**, 644.

⁵ Parrot, J., and Lavollay, J., *Compt. Rend. Acad. Sc., Paris*, 1944, **218**, 211.

⁶ Chambers, R., and Zweifach, B. W., *Physiol. Rev.*, 1947, **27**, 436.

⁷ Duran-Reynals, F., *Bact. Rev.*, 1942, **6**, 197.

⁸ Wislocki, G. B., Bunting, H., and Dempsey, E. W., *Am. J. Anat.*, 1947, **81**, 1.

⁹ Meyer, K., and Ragan, C., *Modern Concepts of Cardiovasc. Dis.*, 1948, **17**, No. 2.

[†] Furnished by the Charles E. Frosst & Co., courtesy Dr. E. Lozinski.

TABLE I.
Area of Spread of Intradermal India Ink (sq. inches).

	Controls		Propylene glycol		Rutin in propylene glycol	
	Ink	Ink-Hy'ase	Ink	Ink-Hy'ase	Ink	Ink-Hy'ase
	.14	1.20	.35	1.14	.11	.40
	.28	1.10	.21	.87	.12	.22
	.44	1.41	.25	.84	.12	.35
	.22	1.20	.24	.45	.07	.38
	.27	2.00	.49	.90	.10	.39
	.25	2.50	.25	1.28	.13	.40
	.22	2.52	.30	.96	.14	.30
	.30	1.92	.28	.98	.14	.43
	.28	2.20	.33	.85	.13	.33
	.28	1.00	.32	.80		
	.35	1.20				
	.10	1.30				
	.17	.59				
	.20	1.05				
Mean	.25	1.51	.302	.907	.117	.355
Standard error of mean	.030	.196	.041	.118	.016	.085
Differences Between Means in Ink-Hyaluronidase Data.						
Mean (controls) — Mean (propylene glycol) = 0.603* ± 0.228						
Mean (propylene glycol) — Mean (rutin in propylene glycol) = 0.552† ± 0.145						
Differences Between Means in Ink Without Hyaluronidase Data.						
Mean (controls) — Mean (propylene glycol) = 0.052 ± 0.050						
Mean (propylene glycol) — Mean (rutin in propylene glycol) = 0.185† ± 0.043						
Analysis of Variance.						
			F (actual)		F (0.01 signif.)	
Ink-Hyaluronidase			19.2†		5.39	
Ink			16.3†		5.39	

* Significant.
† Highly significant.

variance it is evident that the results are due to rutin treatment and not variations within the individual animals. Propylene glycol, which was used as the rutin solvent, inhibits the spreading power of hyaluronidase and this inhibition is increased by a highly significant factor when rutin is also present. Propylene glycol produces slight shock and the resultant liberation of adrenalin probably is responsible for inhibition of hyaluronidase activity. Favilli¹⁰ and Homburger¹¹

have shown that adrenalin has this property. *Conclusions.* Rutin markedly inhibits the spreading activity of intradermally injected inked hyaluronidase. The spread of control injection of ink is also inhibited. Propylene glycol exerts a similar but less intense effect on hyaluronidase and does not reduce the spread of control injections of ink.

¹⁰ Favilli, H., quoted in ¹¹.
¹¹ Homburger, F., *Yale J. Biol. Med.*, 1944-45, **17**, 479.

Rutin in Histamine Shock.*

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The vitamin P substances are a closely related group of compounds which were introduced by Armentano and Szent-Gyorgyi¹ and co-workers as capillary stabilizers. The increased capillary fragility in allergic states led to the study of their effects in allergic diseases and histamine shock. The published findings are contradictory. Jersild,² who presented the first of the anti-allergic reports, claimed dramatic improvement when a patient with severe Henoch-Schonlein's purpura was given vitamin P. More recently, Davis,³ without giving details, stated that vitamin P had no effect on this disease. Kugelmass⁴ reported two cases of allergic purpura which responded favourably to vitamin P and Madison and Pohle,⁵ in a preliminary note, have indicated that rutin has some beneficial effect in this disease. Rappaport and Klein⁶ claimed to have reversed by means of calcium eriodictate, the abnormal capillary fragility seen in allergic children. Unfortunately they failed to mention the effects, if any, on the associated clinical allergic manifestations. In the guinea pig, protection against serum anaphylaxis by hesperidin, has been claimed by Hiramatsu⁷ and confirmed by Raiman, Later and Necheles,⁸ using rutin. In a report of preliminary

studies, Wilson, Mortarotti and De Eds,⁹ employing rutin, could find no such anti-histamine effect in the guinea pig, when an M.L.D. of histamine was used. Wilson *et al.*⁹ thought that rutin did exert an antihistamine effect when the dose of histamine was L.D. 50. However they had pretreated their animals with a vitamin P-free diet. As Parrot and Richet¹⁰ have shown, this diet renders guinea pigs more susceptible to histamine and a dose of histamine which is L.D.50 for normal guinea pigs, may become L.D.100 for P-deficient animals. The effect of rutin therefore may have been the restoration of histamine sensitivity to normal and the findings cannot be interpreted with respect to normal animals. This information became available to Wilson *et al.* after their experiments were completed. Finally, Ambrose and De Eds¹¹ have shown that intravenous rutin delays the appearance of intravenous trypan blue in histamine induced skin wheals.

The doses of rutin administered by Wilson *et al.*⁹ varied up to 10 mg and Raiman *et al.*⁸ 2 mg. In the present experiments, the possible anti-histamine effects of large doses of rutin[‡] were studied in rabbits. The rutin was given orally, in tablets, or parenterally in propylene glycol solution or emulsion. In one series, repeated injections of rutin preceded the histamine. The M.L.D. for intravenous histamine dihydrochloride[§] was found

* Aided by a grant from the Cooper Fund of the Faculty of Medicine, McGill University.

[†] Fellow in Medicine, American National Research Council.

¹ Armentano, L., Bentsah, A., Beres, T., Rusznyak, S., and Szent-Gyorgyi, A., *Deutsch. Med. Wschr.*, 1936, **62**, 1325.

² Jersild, T., *Lancet*, 1938, **234**, 1445.

³ Davis, F., *Blood*, 1948, **2**, 129.

⁴ Kugelmass, I. N., *J.A.M.A.*, 1940, **115**, 519.

⁵ Madison, F. W., and Pohle, H. W., *J. Lab. Clin. Med.*, 1947, **32**, 340P.

⁶ Rappaport, H. G., and Klein, S., *J. Paed.*, 1941, **18**, 321.

⁷ Hiramatsu, N., *Jap. J. Derm. Urol.*, 1941, **49**, 304, quoted in ⁸.

⁸ Raiman, R. J., Later, E. R., and Necheles, H., *Science*, 1947, **106**, 368.

⁹ Wilson, R. H., Mortarotti, T. G., and De Eds, F., *J. Pharmacol.*, 1947, **90**, 120.

¹⁰ Parrot, J., and Richet, G., *Compt. Rend. Soc. Biol.*, 1945, **139**, 1072.

¹¹ Ambrose, A. M., and De Eds, F., *J. Pharmacol.*, 1947, **90**, 359.

[‡] Furnished by the Charles E. Frosst & Co., courtesy Dr. E. Lozinski.

[§] Furnished by the Hoffman La-Roche Co., courtesy Mr. P. Blanc.

TABLE I.
Effect of Single Injections of Rutin on Histamine Shock.

No.	Wt, lb	Sex	Rutin, mg	Propylene glycol, cc	Route	Interval prior to histamine	Death
1	3.75	F	100	1	i/v	20 min.	Within 15 min.
2	2.75	F	100	1	i/p	30 "	"
3	7.5	F	100	1	"	3 hr	"
4	5.5	M	100	1	"	5.5 "	"
5	5.9	F	2000	10	"	22 min.	"

TABLE II.
Effect of Multiple Injections of Rutin in Histamine Shock.

No.	Wt, lb	Sex	Rutin, mg	Amount and type propylene glycol medium		Route	Interval prior to histamine		Result	
1	6.6	M	400	2.0	cc soln.	i/m	30	min.	Death within 15 min.	
			"	"	"	"	"	"		"
			"	"	"	"	"	15		"
			"	"	"	"	"	"		"
			1600							
2	3.75	F	100	5.0	" 10% emuls.	i/p	9	hr	"	
			100	1.5	" soln.	"	8	"		
			150	2.25	" "	"	6	"		
			100	1.0	" 33% emuls.	"	5	"		
			200	5.0	" 10% "	"	3	"		
			200	1.0	" 50% "	i/m	"	"		
			850							
3	5.25	F	100	1.0	" 33% "	i/p	9	"	"	
			200	3.0	" soln.	"	8	"		
			40	0.4	" "	i/v	6.75	"		
			60	0.6	" "	i/m	"	"		
			100	1.0	" 66% emuls.	"	5	"		
			200	1.0	" 50% "	"	3.25	"		
			200	"	" " "	i/p	"	"		
			900							
4	4.75	F	100	2.0	" soln.	i/v	25	"	"	
			200	3.0	" 33% emuls.	i/p	23	"		
			100	1.0	" 66% "	i/m	22	"		
			100	"	" " "	i/p	"	"		
			200	"	" 50% "	"	20	"		
			200	"	" " "	i/m	"	"		
			900							
5	5.5	F	100	1.0	" 33% "	i/p	72	"	"	
			200	3.0	" soln.	"	71	"		
			100	1.5	" "	"	69	"		
			100	1.0	" 66% emuls.	i/m	68	"		
			200	"	" 50% "	"	66	"		
			200	"	" " "	i/p	"	"		
			900							

At autopsy, Animals 2, 3, and 4 showed deposits of rutin at the injection sites.

to be 0.75 mg per lb. in 12 rabbits and this dosage level and the intravenous route used in all parenterally rutinized rabbits.

Previous studies in guinea pigs^{8,9} have indicated that the anihistamine and anti-anaphylactic effects were confined to a brief

period some 10 to 30 minutes following administration of rutin. On this account, in the first group of rabbits, (Table I, No. 1, 2, 5; Table II, No. 1) the time interval between injections of rutin and histamine varied between 15 and 30 minutes. Under these circumstances no protection was observed, with up to 2000 mg of rutin. To exclude the possibility that the antihistamine effect of rutin in the rabbit is characterized by a long latent period (as is seen clinically in the treatment of abnormal capillary fragility states) an unstable emulsion of rutin was injected in addition to the solution and these were given repeatedly. The persistence of rutin deposits in 3 of 4 rabbits so

treated, would appear to indicate that an extended absorption had taken place. Even with this procedure no antihistamine power could be found.

The complete results are presented in tabular form. In addition, 2 rabbits given 50 mg of rutin daily orally for 24 days, failed to survive a 0.5 mg per lb dose of intravenous histamine.

Summary and Conclusions. From the tables it can be seen that pretreatment with large doses of rutin, up to 2000 mg failed to protect rabbits against a minimum lethal dose of histamine. It therefore appears that in the normal rabbit, rutin is devoid of noteworthy antihistamine properties.

16556

Mouse Leukemia. XIII. A Maternal Influence that Lowers the Incidence of Spontaneous Cases.

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The discovery¹ of the milk factor as the cause of the difference in reciprocal hybrids between mouse strains of high and low incidence mammary cancer has naturally led to the search² for a similar agent to account for parallel differences between reciprocal hybrids between strains of high and low incidence of leukemia.³ But evidence of a specific maternal factor inducing leukemia was

not found. The question remains, what mechanism is responsible for the differences in reciprocal F₁ hybrids?

In the following experiment a high-leukemic strain C58, gens. 59-61, brother x sister, was crossed with a low-leukemic strain StoLi, gens. 54-57, brother x sister. (In a cross between such highly inbred strains, all first generation hybrids have the same genetic constitution.) The incidence of spontaneous leukemia was observed in 6 classes of F₁ females: namely, from reciprocal matings, fostered by nurses of strain C58, StoLi, or Balb. Each litter was divided among foster nurses of the 3 strains without receiving a drop of milk from its own mother. Each parturition was witnessed by one of a team of 4 observers[†] who maintained an uninterrupted, night and day vigil of each pregnant

* Diagnoses of cases doubtful in gross were contributed by Dr. M. N. Richter and Dr. R. A. Miller with technical assistance from Miss L. Lewis.

¹ Little, C. C., *et al.*, *Science*, 1933, **78**, 465; Bittner, J. J., *Am. J. Cancer*, 1940, **39**, 104.

² Barnes, W. A., and Cole, R. K., *Cancer Res.*, 1941, **2**, 99; Furth, J., Cole, R. K., and Boon, M. E., *Cancer Res.*, 1942, **2**, 280; Kirschbaum, A., and Strong, L. C., *Proc. Soc. Exp. Biol. and Med.*, 1942, **51**, 404.

³ MacDowell, E. C., in *Year Book*, Carnegie Inst. of Wash., 1934, **33**, 42; MacDowell, E. C., and Richter, M. N., *Arch. Path.*, 1935, **20**, 709.

[†] The authors, with the highly appreciated cooperation of Dr. Vernon Bryson and Miss Elsie Ward.

female from the 18th day after the observed mating plug. Each new-born mouse was removed from the box after the mother had cleaned it up as much as she would before the next one appeared. When the interval between successive births was prolonged, as occasionally happened, the new-born mouse was replaced by one or more from the Balb strain (impossible to confuse with the black-eyed hybrids), to focus the mother's activity until the next birth. The litter was not distributed to the nurses until the last one was born. Even though the last birth might not occur for several hours after the first, no difficulty was encountered, provided the new-borns were thoroughly warmed up when offered to the nurses and all traces of blood carefully washed off by the observer, if not by the mother. Every nurse had been suckled by other young for 4-24 hours before the experimental animals were presented, so that none of the experimental animals received the first milk. StoLi and C58 mothers to be used as nurses were temporarily given Balb new-borns to draw off the first milk and to maintain nursing behavior until needed. Each nurse raised 5, or, in a few cases, 6 young, the number being increased to 5 when necessary by Balb new-borns. On the 28th day after birth each litter was weaned and distributed to permanent boxes, each box housing one mouse from each of the 6 classes.

Results. The occurrence of leukemia in each of the 6 classes is given in Table I. The curves in Fig. 1 represent the total leukemic deaths that had accumulated at

successive 50-day periods as per cents of the total number in the class. The 3 deaths between weaning and 6 months have been omitted. As in the figure, the 6 classes will be identified by the initial of the mother's strain followed by that of the nurse's strain.

The role of B nurses provides the key to the interpretation. This experiment was actually undertaken to verify the seemingly direct influence of nurses of this strain in raising the incidence of leukemia unexpectedly observed in an experiment designed as a test for genetic segregation.⁴ But long before the present experiment was concluded, further analysis of the previous data showed that the B nurses had no effect upon leukemia as such, but they had increased the resistance to an entirely unrelated disease to which males alone were susceptible. As a result, the longer lives of males with B nurses gave more opportunity for leukemia to appear. Thus, a seeming sex influence on leukemia was eliminated. The present results confirm the conclusion that nurses of this strain have no influence upon leukemia. B nurses give virtually the same results as the nurses of the mother's strain. That is, with C mothers, B and C nurses give the same result, and with S mothers B and S nurses give the same result. In the first case the B nurses are actually higher and the second actually lower than the nurses of the mother's strain, but such inconsistent deviations appear to have no significance. If B nurses are neutral, C-B, and S-B indicate the full effects of the purely maternal factor, which thus is responsible for highly significant differences in the proportion of leukemics and in their longevity. It follows, also, that nurses of the mother's strain do not augment this influence from the mother herself. This does not mean that C and S nurses are neutral, for with mothers of the opposite strain (C-S and S-C) they completely reverse the maternal influence upon leukemia and less fully modify the maternal influence upon longevity. Accordingly, within each of these 2 nurse strains, the mother's strain would be supposed to have no influence upon leukemia, but only upon longevity.

TABLE I.
Incidence of Leukemia and Average Length of Life of F₁ Females According to the Strain of the Mother and of the Nurse. Parentheses = Total Number of Mice.

Mother-nurse strain	Leukemics, %	Length of life, avg—days	
		Leuk.	Neg.
C-C	85.3 (34)	575.7	519.0
C-S	55.9 (34)	733.4	740.6
C-B	93.7 (32)	568.9	—
S-C	80.0 (30)	771.2	747.1
S-S	54.8 (31)	855.6	850.1
S-B	50.0 (28)	765.6	799.0

C nurses move the whole longevity curve for S mothers (S-S) nearly half-way towards the C mothers' curve (C-C). S nurses move the curve for C mothers more than 2/3 of the way toward that for S mothers. The fact that the nurse can entirely reverse the maternal influence upon the proportion of leukemics and only partially reverse this influence on longevity suggests that the association between longer lives and fewer leukemics may not be directly dependent. Indeed, the longevity of non-leukemics follows that of leukemics so closely (Table I) that this longevity factor appears to be non-specific, resisting all causes of death, and thus unable to account for the modification of the proportion of leukemics.

The foregoing analysis indicates a strain differential in the maternal contribution of a non-specific influence upon longevity and a specific influence upon the incidence of leukemia. Further, it may safely be supposed that the mechanisms responsible for these maternal influences are basically the same as those responsible for corresponding influences transmitted by nurses. But it does not necessarily follow that the higher incidence of leukemia from C mothers is dependent upon a positive leukemia-favoring factor, parallel to the virus-like milk factor in mammary cancer of mice. The action of the S nurses with C mothers gives evidence that the S effect is positive resistance and not merely the absence of a possible pro-leukemia influence from a C nurse, for C-B shows that the absence of a C nurse gives no such reduction in the proportion of leukemics.

Further evidence that the S mother influence is positive resistance to leukemia appeared in the experiment previously cited.⁴ In this experiment the heredity from strain C58 was carried exclusively by males. The critical 2nd back-cross, included 50 families of about 50 mice each, all born to inbred S mothers and a different 1st backcross father for each family. These families varied in the incidence of leukemia from 0% to 41%. Considering the quarter of

these families with the highest total leukemia, the highest incidence appeared in the mice with youngest mothers; with increasing age of the mother at parturition the per cent of leukemics was increasingly reduced until it was almost eliminated. The next lower quarter of the families showed the same declining incidence with increasing age of mother, but the maximum incidence from the youngest mothers was necessarily lower. As this maximum was further lowered in the next quarter of the families the effect of mothers' increasing age was less obvious and in the quarter of the families with only 0%-10% leukemics no change with mother's age could be recognized. A maternal factor becoming increasingly potent with increasing age of the mother at parturition was responsible for the highly effective suppression of inherited tendencies to leukemia.

This progressive reduction in the frequency of leukemics was accompanied by increasing length of life. Since the longevity of non-leukemics in all the 50 families taken together showed no correlation with mother's age, the declining incidence of leukemia was

TABLE II.
Distribution of Leukemic and Non-leukemic Deaths of F₁ Females from Young and Old S Mothers Which Nursed Their Own Offspring. 50-Day Classes Represented by Low Limit.

Length of life, days	Mothers			
	Young		Old	
	Leuk.	Neg.	Leuk.	Neg.
300	1	1		
350	4	1		
400	5	—		
450	5	2		
500	8	0		1
550	9	4	2	2
600	10	1	1	2
650	7	1	5	3
700	4	1	6	8
750	4	—	4	4
800	1	1	4	6
850	4	1	7	6
900			13	4
950			3	1
1000			3	—
1050			1	1
1100			1	
	% L	82.6 (75)	56.8	(88)

Diff. 25.8 \pm 2.17. $\chi^2 = 13.8$. $P = .0002$.

⁴ MacDowell, E. C., Potter, J. S., and Taylor, M. J., *Cancer Res.*, 1945, **5**, 65.

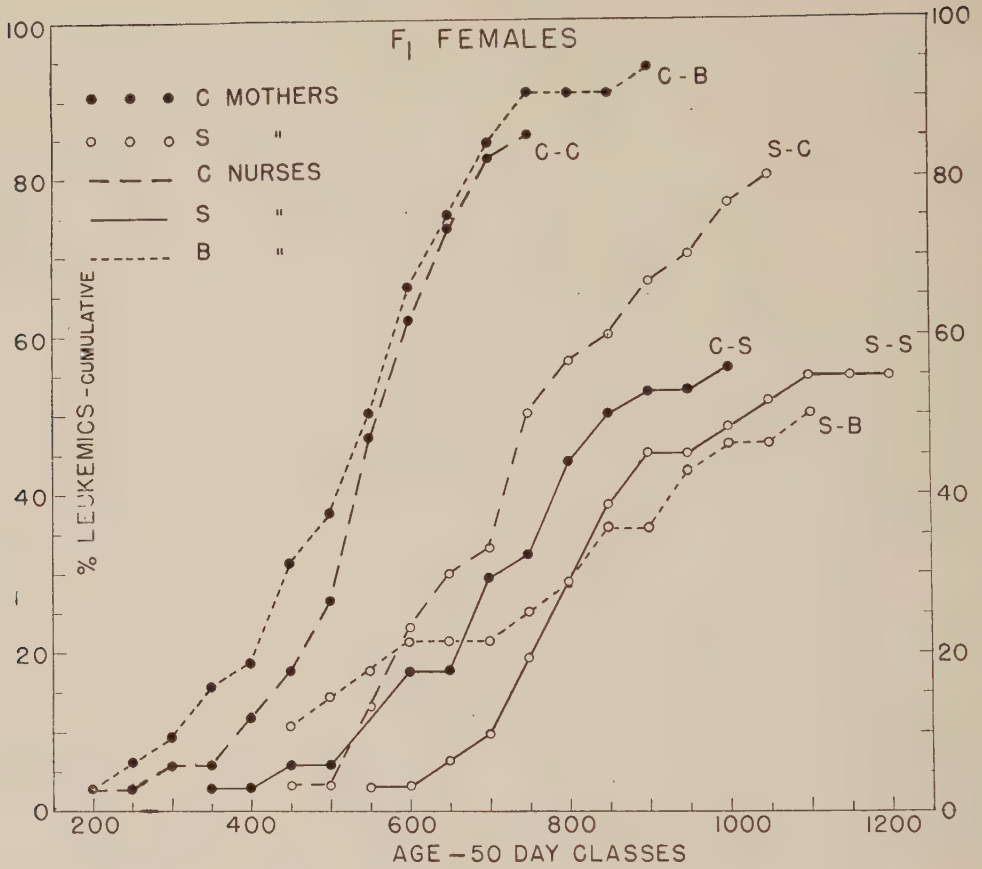


FIG. 1.
Strain of mother vs. strain of nurse. Leukemic deaths of F_1 females accumulated at successive age periods, given as % of total number of mice, according to the strain of the mother (first initial) and of the nurse (second initial): C = C58, S = Storrs-Little (StoLi), B = CSH Bagg albino (Balb); age classes designated by low limit.

at that time interpreted as a secondary result of the maternal factor specifically delaying the appearance of leukemia. Considering the evidence of non-specific maternal influence upon longevity given above and to follow, this interpretation appears now to be untenable. In the 2nd back-cross families an extremely complex situation was created by the simultaneous segregation of genetic factors for leukemia and for non-specific longevity. These probably confounded what now appears, under far more simple conditions, to be a non-specific maternal influence on longevity.

A special experiment was set up to determine the influence of older S mothers under more simple conditions. As in the nursing

experiment the test animals were F_1 females; one group from young and one from old StoLi mothers. Each mother nursed her own young. In order to obtain reliable reproduction with old mice it is necessary to start breeding them while young. The S females to serve as old mothers were continually mated with their sibs. At the appropriate time their daughters were saved to serve as young mothers. When the old females were 29 weeks old and the young females 7 weeks old they were mated to C58 males with 3 young and 2 old females in each mating. The offspring were housed, 5 of the same class per box, boxes from young and old mothers alternating on the shelves.

Table II, Fig. 2 give the primary results.

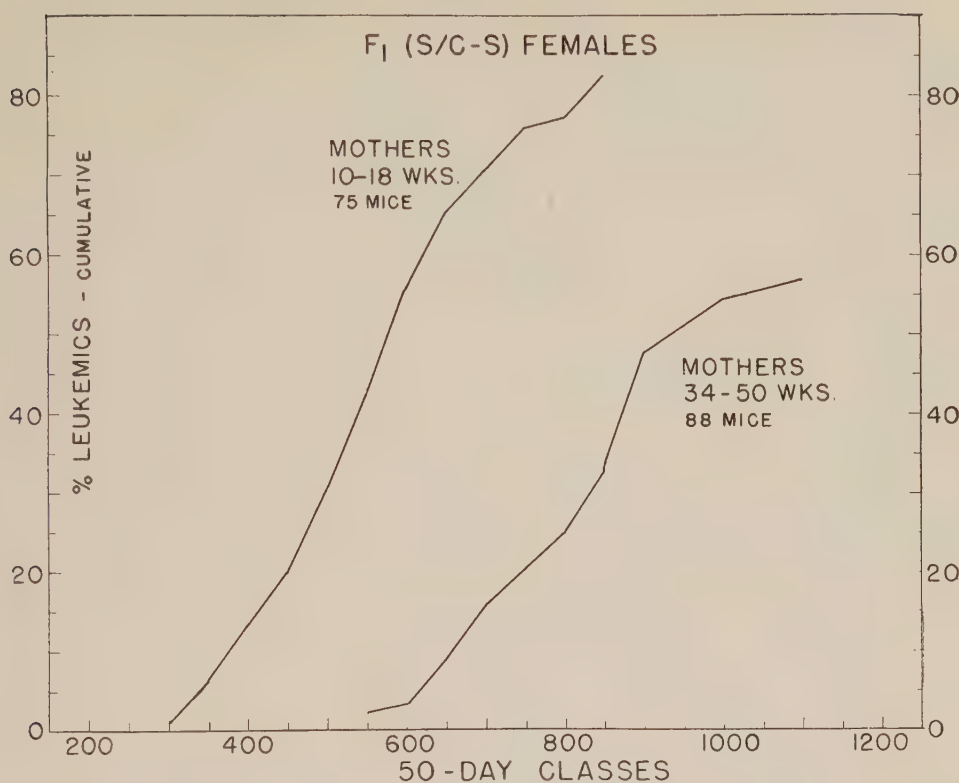


FIG. 2.

Young vs. old S mothers. Leukemic deaths of F_1 females nursed by own mothers. Presentation as in Fig. 1.

Mothers 34 weeks and older unquestionably reduce the incidence of leukemia and lengthen the lives of leukemics. The first leukemic death of mice from young mothers occurred about 250 days before the first leukemic death from old mothers (550-day class) at which time 42% of all the young from young mothers had died with leukemia. At 850 days when the last deaths of mice from young mothers gave a final 82% leukemics, 33% of the mice from old mothers had died with leukemia. Here again the close association of less leukemia and longer lives is evident, but here also the longevity of non-leukemics as well is influenced. Non-specific factors delaying all causes of death will not account for modification of the incidence of leukemia. Fig. 3 shows the cumulative %-distribution of deaths of negatives and of leukemics. The negatives from young mothers coincide with the leukemics. The negatives from old

mothers are actually slightly earlier than the leukemics, but in face of the difference for the negatives from young mothers this difference is probably insignificant. A curve for the distribution of leukemic deaths among 40 inbred C58 females (mothers from nursing exp.) is added to show another break in the longevity-leukemia association, for the F_1 females even with young S mothers live considerably longer than inbred C58s, although the incidence of leukemics is virtually the same. To check the reliability of the difference between young and old mothers and to find possible minor effects within the young and old mother classes, each class has been subdivided (Fig. 4). The two curves from young mothers give the same incidence of leukemia, but those from the 15-18 wk.-females lived slightly longer. The two curves from old mothers show the same longevity, but the final incidence of leukemia is slightly

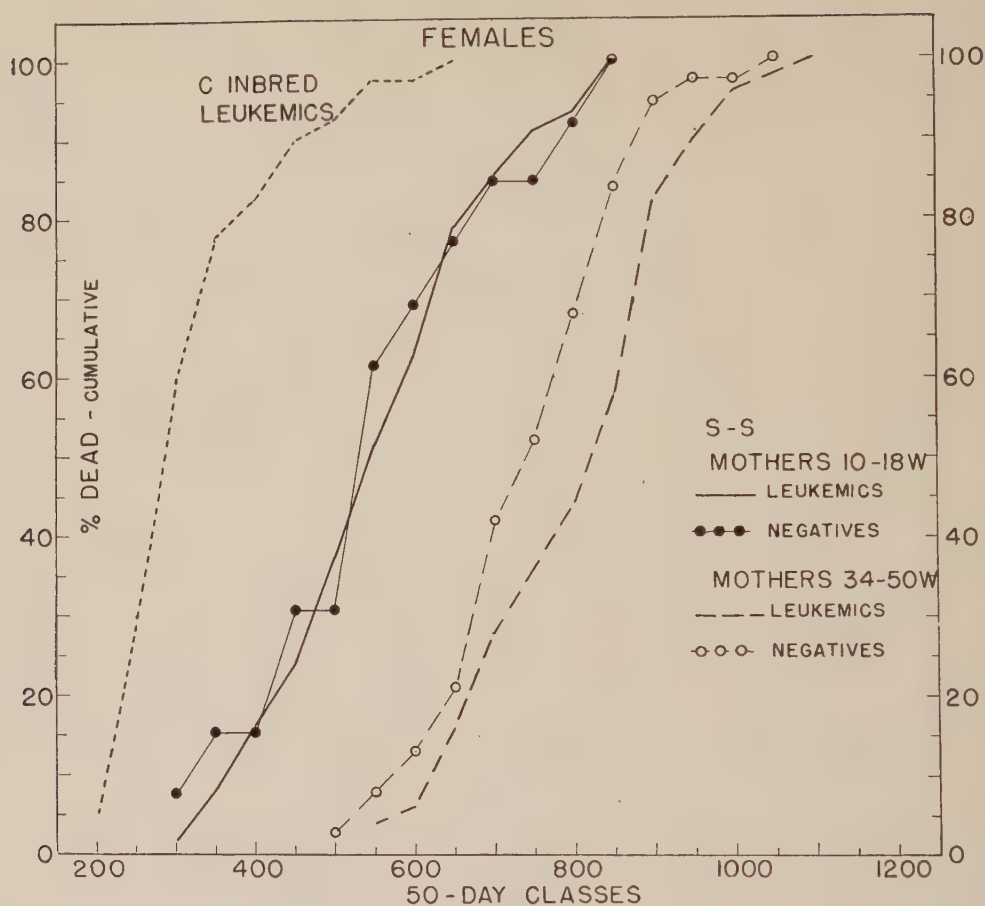


Fig. 3.

Young vs. old mothers. Age distribution of leukemic and of non-leukemic deaths of F_1 females as cumulative % of the final number with each diagnosis. Also, corresponding age distributions of leukemic deaths of inbred C females.

lower for the older mothers. These differences between sub-classes may be suggestive but not significant; each half of the data gives essentially the same result.

The bearing of this experiment upon the nursing experiment is made clear by superimposing the nursing experiment curves for S-S (open circles) and C-C (solid dots) upon Fig. 4. The virtual identity of the S-S curves in the two experiments indicates that the S females in the nursing experiment were "old", (as the records show them to have been) and justifies the interpolation of the term 'S mother's age factor' in the interpretation proposed for the results of the nursing experiment. From that experiment it becomes evident that the S mother's age in-

fluence may be transmitted before birth, and it may also be transmitted through the nursing (milk) alone.

The agreement between the young mothers' curves and the C-C curves indicates that the difference between reciprocal F_1 hybrids between strains C58 and StoLi disappears when the resisting influence of old S mothers is not present. C58 males contribute as strong a leukemic tendency to F_1 hybrids as C58 females and in these experiments this appears to be virtually as strong as in inbred C58, in other words full dominance.

Conclusion. The maternal influence responsible for a difference in the incidence of leukemia in reciprocal F_1 hybrids between a high—(C58) and a low leukemic strain

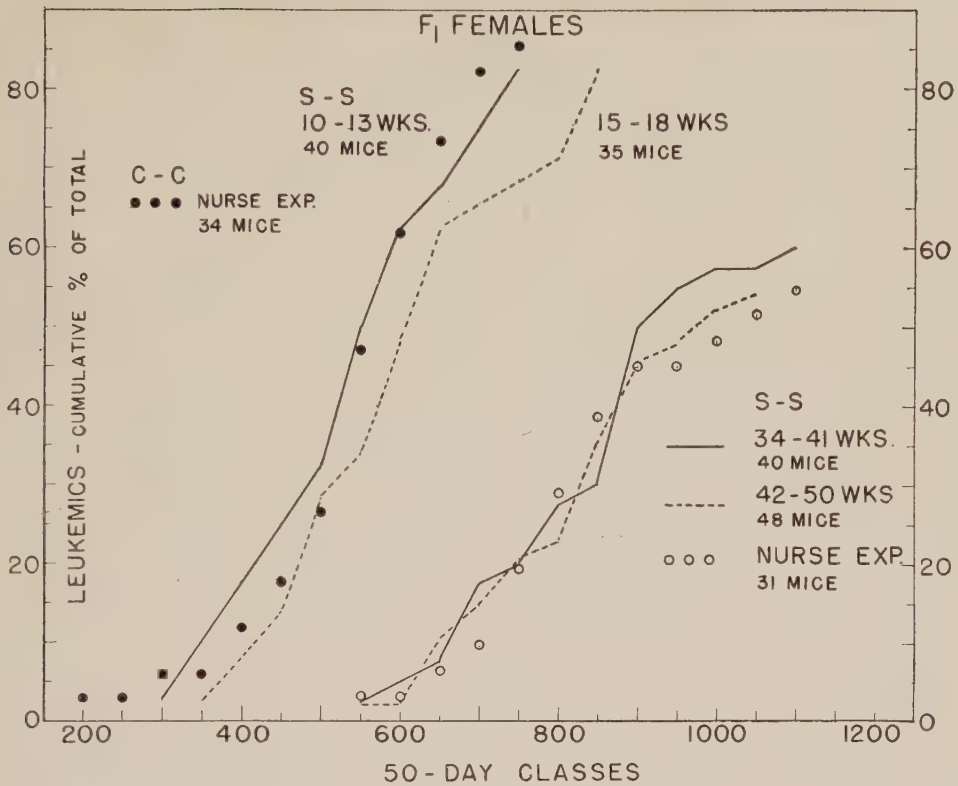


FIG. 4.

Young vs. old S mothers. Data of Fig. 2 with young and old mothers' groups subdivided. Also, curves from Fig. 1 for C-C and S-S represented by unconnected points.

(StoLi) consists of a definite resistance to leukemia contributed by the low strain mother. This maternal resistance may be contributed before birth or through nursing alone. It is not found at earliest sexual maturity, but becomes increasingly potent with advancing age. When this resistance is

absent the leukemic heredity of C58 shows full dominance, whether introduced by a father or mother. An influence resisting all causes of death non-specifically is similarly transmitted by StoLi mothers as their age increases.

Comparative Cortical Action of Different Pregnenolones in the Adrenalectomized Guinea Pig.

SILVIO BRUZZONE AND HUGO LOPEZ. (Introduced by Å. Lipschutz.)

From the Department of Experimental Medicine, National Health Service of the Republic of Chile, Santiago.

The adrenalectomized rat,^{1,2} dog,³ and guinea pig⁴ can be kept alive for a certain time with 21-acetoxypregnenolone (abbreviated 21-AOP; Δ^5 pregnene-3,21-diol-20-one-21 acetate). In the present work additional results with 21-AOP and results with 2 other compounds will be given: with pregnenolone (abbreviated P; Δ^5 -pregnene-3-ol-20-one and its 3-acetate (abbreviated 3-AP). The compound P has been found to be active in the adrenalectomized rat by Selye,¹ but not by Segaloff and Nelson.² The second compound 3-AP has so far not been used in adrenalectomized animals.

Experiments. Ablation of the adrenals has been carried out in 2 stages by the technique we have described previously.⁴ Pellets of the steroids were implanted subcutaneously after completion of the ablation of the second adrenal. It was necessary to implant 10 to 25 pellets so as to obtain absorption of sufficient quantities. Results with P and 3-AP are summarized in Table I. Experiments with 21-AOP (18 animals from our former work⁴ and 7 new animals) are included for comparison. Animals which died within 2 days after the second stage of operation are not included; neither were animals in which an adrenal fragment was found at necropsy.

Adrenalectomized guinea pigs receiving no steroid seldom survive more than 7 days and rarely for 9. As seen from the table, with P only one animal survived for 9 days. On the other hand, with 21-AOP no less than

80% survived for more than 9 days. Animals receiving 3 AP occupy an intermediate position; several animals survived from 9 to 19 days. This was not due to the greater quantities of 3-AP administered since in 3 out of the 6 surviving animals there was absorption of 906 to 1060 μ g per day, *i.e.* still in the range of the experiments with P and 21-AOP. There was loss of weight in all the 3 groups.

Our findings with P show that as much as 1.5 mg per day, *i.e.* a quantity twice that of 21-AOP did not prolong the survival of our guinea pigs. This corroborates the former findings with P in the rat.² Though P has had no influence on survival of the animals there was apparently some beneficial action. In this group no premortal convulsions have been observed as is the rule in guinea pigs dying from adrenal insufficiency. Though convulsions may have been overlooked, no hyperextension of the extremities was observed in these dead animals as is frequently the case with dead untreated animals.

A comparison between the groups receiving 21-AOP and 3-AP respectively is of considerable interest. There is, first, the remarkable difference as to the percentage of survival. Secondly, the maximal length of survival with 21-AOP was greater than with 3-AP. Third, the loss of weight was less pronounced with 21-AOP than with 3-AP. Fourth, with 21-AOP the chance of survival increased with greater quantities administered; on the contrary, as already stated, with 3-AP there was apparently no greater chance of survival when greater quantities were given.

Our comparative results show that 3-AP is a very poor substitute for cortical ste-

¹ Selye, H., *Science*, 1941, **94**, 94.

² Segaloff, A., and Nelson, W. O., *Endocrinology*, 1942, **31**, 592.

³ Cleghorn, A. R., *Endocrinology*, 1943, **32**, 165.

⁴ Bruzzzone, S., Borel, H., and Schwarz, J., *Endocrinology*, 1946, **39**, 194.

TABLE I.
Fifty-seven Adrenalectomized Guinea Pigs with Subcutaneously Implanted Tablets of 3 Different Pregnenolones.

Steroid	Absorbed per day, μg	No. of animals	No. of animals dying on days indicated			% of animals surviving for more than 9 days	Longest survival, days	Loss of wt per day
			2-7	8-9	>9			
Pregn.	716-1466	10	9	1	0	0	9	113
3-acetate	733-2625	22	14	2	6	27	19	140
21-acetoxy	700-1250	25	5	0	20	80	162	65

Prgn. M.P. = 190°; 3-acet. pregn. M.P. = 149-150°; 21-acetoxy-pregn. M.P. = 183°.

roids. But since 3-AP is more active than P one wonders whether the cortical action of 21-AOP might be further increased by esterification at C₃ also, *i.e.* using a diester of 21-oxypregnenolone.

Summary. Pregnenolone was unable to keep alive the adrenalectomized guinea pig. There was some cortical activity with 3-

acetate-pregnenolone. But the activity of 3-acetate of pregnenolone was much less than that of 21-acetoxypregnenolone.*

* Our thanks are due to Dr. E. Oppenheimer, Ciba Pharmaceutical Products, Summit, N.J., and Dr. Carl Miescher, Ciba, Basel, for a generous supply of steroids.

16558

On the Antitumorigenic Action of Large Quantities of Pregnenolones.

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Estrogen-induced abdominal fibroids can be prevented by different 3-keto-steroids,¹ of which progesterone is the most powerful.² But the original supposition that there is a correlation between progestational and antifibromatogenic action^{2,3} had to be discarded when it was shown that enhancement of progestational activity by a substitution at C₁₇ is not accompanied by an increase of antifibromatogenic activity; ethinyl- and ethyl-testosterone are even less antifibromatogenic than testosterone.^{4,5}

So far no steroid with an hydroxyl group at C₃ has been found to be antifibromatogenic; neither 21-acetoxypregnenolone (abbreviated 21-AOP),³ nor 3-acetate-pregnenolone (abbreviated 3-AP)⁶ prevented estrogen-induced fibroids even when quantities several times larger than those of progesterone were used. On the other hand, pregnenolone derivatives may show unexpected activity when administered in very large quantities; progestational⁷ and cortical activity^{8,9,10} and protective action on the sem-

¹ Lipschutz, A., *Nature* (Lond.), 1944, **153**, 260; *Experientia* (Basel), 1946, **2**; *Bull. Acad. Méd.* (Paris), 1947, 229.

² Lipschutz, A., Bruzzzone, S., and Fuenzalida, F., *Cancer Research*, 1944, **4**, 179.

³ Lipschutz, A., Bruzzzone, S., and Fuenzalida, F., *Proc. Soc. Exp. Biol. and Med.*, 1943, **54**, 303.

⁴ Iglesias, R., and Lipschutz, A., *Lancet*, 1946, **2**, 488.

⁵ Lipschutz, A., Iglesias, R., Bruzzzone, S., Fuenzalida, F., and Riesco, A., *Fourth Internat. Cancer Research Congress*, 1947, 23; *Texas Reports of Biology and Medicine*, 1948, in publication.

⁶ Iglesias, R., unpublished work; see series LXXXI in Table I of the present paper.

⁷ Selye, H., and Masson, G., *J. Pharm. and Exp. Therap.*, 1943, **77**, 3.

⁸ Selye, H., *Science*, 1941, **94**, 94.

inal tubules have been observed.¹¹ For all these reasons we decided to use in experiments with estrogen-induced fibroids, much larger quantities of 21-AOP and 3-AP than those we have formerly used.

Experiments. Pellets of 21-AOP and 3-AP were implanted beneath the skin into castrated female guinea pigs; absorption per day varied according to the number of pellets implanted. A pellet of α -estradiol was also implanted into the same animals. Control animals were implanted with a pellet of α -estradiol only. Results are summarized in Table I.

With subcutaneously implanted pellets of α -estradiol an average fibrous tumoral effect of F.T.E.=5 to 6 was obtained in 90 to 99 days. The simultaneous absorption of 70 of 322 μ g of 21-AOP per day did not alter, or altered only very slightly, this result.³ There was still a remarkable tumoral effect with such quantities as 808 to 1241 (Table I), though the F.T.E. was somewhat diminished. There was no antifibromatogenic action at all with 114 to 506 μ g of 3-AP per day; but with larger quantities of 3-AP there was a definite preventive action the F.T.E. dropping from 5 to 1.6 and 1.3. No animal receiving 552 to 1670 μ g reached the average F.T.E. of the control group though some may approach it (see range); especially remarkable was the fact that the incidence of fibroids of about 3 to 6 mm in diameter (Class 2 and 3) dropped with large quantities of 3-AP from 2 to 0.1.

The fact that 3-AP was more antifibromatogenic than 21-AOP is all the more remarkable as the cortical activity at 3-AP was considerably less¹⁰ than that of 21-AOP.⁹

Other comparative findings with large quantities of the 2 pregnenolones also may be mentioned. Uterine bleeding, which is induced in guinea pigs by continuous action of estrogens and prevented by 3-keto-steroids, occurred in the present experiments

TABLE I.
Seventy-two Castrated Female Guinea Pigs with Subcutaneous Implantation of Pellets.

No. of series and duration days	Pregnenolone absorbed per day, μ g	α -Estradiol absorbed per day, μ g	No. of animals	Avg fibrous tumoral effect F.T.E.*	No. of animals with F.T.E. no less than 5*	Incidence of tumoral marks of classes 2 and 3 per animal	Avg uterine wt, g
XCVIII	0	17.47	10	5.1 (1.0-9.0)	5	1.7	5.3 (2.6-10.7)
XCVIII	21-AOP† 808-1241	30.55	14	3.0 (1.5-5.5)	2	0.9	4.2 (2.0-10.6)
CXXXI	0	51.79	10	6.4 (1.5-10.0)	7	2.0	6.5 (2.5-14.5)
LXXXI	3-AP‡ 114-506	36.72	19	5.2 (0.5-10.0)	8	1.8	4.8 (2.5-13.6)
CXXXI	3-AP 552-980	14.97	10	1.6 (1.0-4.5)	0	0.2	2.2 (1.2-3.5)
CXXXI	3-AP 1040-1670	40.103	9	1.3 (1.0-3.0)	0	0.1	2.1 (1.4-2.9)

⁹ Bruzzone, S., Borel, H., and Schwarz, J., *Endocrinology*, 1946, **39**, 194.
¹⁰ Bruzzone, S., and Lopez, H., *Proc. Soc. Exp. Biol. and Med.*, 1948, **68**, 578.
¹¹ Masson, G., *Am. J. Med. Sc.*, 1946, **212**, 1.

* For the explanation of these units see especially 2.
† M.P. = 183°. Pellets 10 to 12 per animal. Surface of the pellet about 60 mm². Absorption was of 1.4 to 1.7 μ g per mm² (avg 1.5 μ g).
‡ M.P. = 149-150°. Pellets 1 to 12 per animal. Absorption per mm² was of 1.5 to 2.8 μ g per mm² (avg 2.2 μ g).

with large quantities of 21-AOP but not with 3-AP. There was only a slight and scarcely significant preventive action against the increase of the uterine weight when large quantities of 21-AOP were given; but with antifibromatogenic quantities of 3-AP the uterine weight dropped considerably.

With 3-AP growth of the mammary glands was rather enhanced, as in former work with 3-ceto-steroids.^{12,13,14} There was some androgenic action with 1040 to 1670 μ g of 3-AP per day as evidenced by the slight enlargement, of the clitoris in 4 out of 9 animals.

The antifibromatogenic action of large quantities of 3-AP was as unexpected as was the estrogenic action of large quantities of androgens.¹⁵ It would be idle to speculate now about the mechanism of similar paradoxical

statements. We wish only to mention that pregnenolone has been tentatively assumed to play a role in the biogenesis of steroid hormones¹⁶ and has been subsequently found in the testicle of the pig.¹⁷

Summary. With quantities of 3-acetate-pregnenolone about 30 to 50 times those of the antifibromatogenic minimum of progesterone, estrogen-induced abdominal fibroids can be prevented in the guinea pig to a very considerable degree. This is the first statement of an antifibromatogenic activity of a steroid with an hydroxyl group at C₃. There was only a slight diminution of the fibromatogenic effect when quantities of 21-acetoxy-pregnenolone as large as 50 to 80 times those of progesterone were given.

Our thanks are due to Dr. Carl Miescher, Ciba, Basel, who suggested work with 3-acetate pregnenolone, for the generous supply of this steroid, and to Dr. E. Oppenheimer, Ciba Pharmaceutical Products, Summit, N.J., for 21-acetoxypregnenolone. Thanks are due to Dr. F. Fuenzalida for the determination of M.P. of the steroids used.

¹⁶ Selye, H., *Rev. Can. Biol.*, 1942, **1**, 577.

¹⁷ Ruzicka, L., and Prelog, V., *Helv. Chim. Acta*, 1943, **26**, 975.

¹² Lipschutz, A., and Vargas, L., *Endocrinology*, 1941, **28**, 669.

¹³ Lipschutz, A., and Zañartu, J., *Endocrinology*, 1942, **31**, 192.

¹⁴ Von Wattenwyl, H., *Follikelhormonapplikation und die hormonale Tumorentwicklung*, Schwabe, Basel, 1944, pp. 177 a. f.

¹⁵ Deanesly, R., and Parkes, A. S., *Brit. Med. J.*, 1936, **1**, 257.

16559

Failure of Oral Saccharine to Influence Blood Sugar.

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A number of investigators have employed saccharine in examining the possibility that a sweet taste results in an alteration in blood sugar level. Various authors have claimed that an increase,^{1,2} a decrease,³ or no significant change^{4,5} in blood sugar followed

drinking a saccharine solution. The most recent report by Kun and Horvath,³ and the only one presenting statistically significant data, shows a drop in blood sugar. We were interested in employing this technique as a means of maintaining a low blood sugar but in the face of earlier conflicting evidence, it was planned first to repeat the work under conditions duplicating as nearly as possible those described by Kun and Horvath.

¹ Syllaba, G., *Guy's Hosp. Rep.*, 1930, **80**, 230.

² Pannhorst, R., *Z. f. klin. Med.*, 1935, **127**, 688.

³ Kun, E., and Horvath, I., *Proc. Soc. Exp. Biol. and Med.*, 1947, **66**, 175.

⁴ Althausen, T. L., and Wever, G. K., *Proc. Soc. Exp. Biol. and Med.*, 1937, **35**, 517.

⁵ Fischer, F., and Schroter, A., *Dtsch. Med. Wschr.*, 1935, **61**, 1354.

TABLE I.
Blood Sugar Values at 10-Minute Intervals After
Oral Administration of Saccharine to Fasting
Subjects.

Subject	10 min.	20 min.	30 min.	40 min.	50 min.
1	110*	98	105	104	102
2	91	98	98	93	97
3	110	107	99	95	101
4	97	95	91	99	97
5	107	108	108	98	104
6	105	102	104	102	99
7	105	106	105	107	109
8	101	97	98	105	106
9	95	96	94	98	95
10	103	101	100	98	95
Mean	102.4	100.8	100.2	99.9	100.5

* Blood sugar values expressed as % of control level.

Methods. The 10 subjects used were departmental personnel who were familiar with the purpose of the experiment and had no apprehension concerning the procedure. All were in good health. On the morning of the test, each subject appeared at the laboratory without breakfast and was made to lie quietly on a cot for 20 to 30 minutes before the first blood sample was taken. Two control blood samples were taken 10 minutes apart and after the second, 50 mg of saccharine in 80 ml of water was drunk over a period of 5 minutes. Ten minutes after the subject began drinking the saccharine solution, the third blood sample was drawn and 4 additional samples were taken at 10 minute

intervals. Duplicate determinations of blood sugar were made on each sample by the micro method of Hagedorn and Jensen.⁶

Results and Discussion. In the accompanying table, the average of the 4 determinations on the first 2 blood samples is taken as the control value. All other values on each subject are then expressed as per cent of his own control. The greatest single variation from a control is 10% and the greatest mean variation for all subjects at any one time period after saccharine is 2.4%.

Why Kun and Horvath obtained consistent and significant lowering of blood sugar and all others, including ourselves, did not, we are unable to explain. The mean control value of all subjects was 97.8 mg%, which is a usual fasting value for the blood sugar as determined by the method used. Therefore, one could not say that other factors raised the blood sugar in our experiments and thus obscured a lowering due to the sweet taste, nor could one account for the absence of a hypoglycemic response in terms of an abnormally low initial blood sugar level.

Summary. Oral administration of saccharine to 10 normal subjects, fasted and at rest, did not influence the blood sugar during the subsequent 50 minutes.

⁶ Hagedorn, H. C., and Jensen, B. N., *Biochem. Z.*, 1923, **137**, 92.

16560

Enzymatic Inactivation of Serum Vasoconstrictor.*

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It has been the practice for many years to abolish the vasoconstrictor properties of serum or defibrinated blood to be used in the perfusion of isolated organs, by passing the blood through oxygenated lungs. The

mechanism of this action is not known.

Our studies on the identification of the substance which causes the vasoconstriction led us to investigate the action of cell-free protein extracts from lung tissue on highly purified preparations of serum vasoconstrictor. The results of this study, which are reported in this paper, indicate that the

* A preliminary report of this paper was published in *Federation Proceedings*, 1948, **7**, 180.

TABLE I.

Preparation of Vasoconstrictor Inactivating Enzyme.

Ground fresh lung tissue (beef or dog) extracted 1 hr with 2 volumes of 1% NaCl. Filtered through gauze.

 Filtrate: brought to $\frac{1}{3}$ saturated $(\text{NH}_4)_2 \text{SO}_4$.
Centrifuged.

 Sediment: suspended in water. Dialyzed 2 days against running tap water at 5°C.
Centrifuged.

 Supernatant solution:
pH adjusted to 5.1.
Centrifuged.

 Sediment: Insoluble,
Active

 Sediment: soluble at pH 7.8,
Active

mechanism by which the lungs inactivate the vasoconstrictor substance may be enzymatic.

Materials and Methods. Serum vasoconstrictor substance was purified as described.¹

Lung extracts were prepared from beef lungs obtained from the slaughter house on the same day the animal was killed. The lung tissue was ground twice and then extracted for one hour at 5° with 2 volumes of cold saline. The mixture was filtered through gauze. Fractionation of the filtrate followed the scheme summarized in Table I.

Dog lungs were treated similarly.

Beef lungs obtained one day after being removed from the animal yielded preparations with only slight activity.

Dog lungs which were chilled rapidly after removal and kept frozen for 3 months gave satisfactory preparations.

The inactivation studies were carried out as follows: Test tubes containing 0.2 ml of a standard serum vasoconstrictor solution (purified material containing 1 mg/ml) 0.2 to 0.5 ml of enzyme solution, and 1.2 ml of 0.05 M phosphate buffer ($\text{K}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4=9$) made up to a total volume of 2.0 ml, were incubated at 37°. 0.1 ml aliquots were withdrawn after varying periods of time, diluted to 1.0 ml, and then assayed in the perfused rabbit ear preparation against a substrate control in the manner described.¹ Substrate con-

trols have never been observed to lose activity. Enzyme controls exerted vasoconstrictor effects representing only about 3% of the initial activity of the incubation mixture.

Results. Under the conditions employed (pH 7.4 to 7.6) the observed inactivation reaction appeared to follow a first order course (Table II).

The effect of pH has been examined at pH 7.2, 7.6, and 8.0. A marked decrease in the rate was observed with decreasing pH, the reaction proceeding about twice as rapidly at pH 8 as at pH 7.2.

The enzyme was found to be heat labile; heating at 60° for 3 minutes resulted in 50% inactivation. Preparations lost activity even when kept cold. The losses have been variable in both chilled and frozen solutions, but, in general, about half the activity was lost in 5 days at 5° C. Keeping the enzyme suspended in 35% saturated ammonium sulfate did not enhance the stability. As

TABLE II.

Inactivation of Serum Vasoconstrictor by Enzyme from Dog Lung. 1.7 mg Protein, 0.1 mg (75 Units) Purified Serum Vasoconstrictor per ml. pH 7.5. 37°C.

Time in hr	% inactivation	$K = \frac{1}{t} \log_{10} \frac{a}{a-x}$
1½	38	23×10^{-4}
3	63	24
4½	69	19
6	77	18

¹ Rapport, M. M., Green, A. A., and Page, I. H., *J. Biol. Chem.*, 1948, **174**, 735.

can be seen from Table I, active enzyme preparations were found in both soluble and insoluble protein fractions. The activity per mg of protein and the stability of the insoluble fraction was usually slightly greater than that of the soluble one. With beef lungs, about $\frac{1}{3}$ of the total activity was found in the soluble fraction; with dog lungs, a smaller fraction of the total activity was soluble.

Summary. Saline extraction of ground lung tissue (beef or dog) followed by ammoni-

um sulfate fractionation, dialysis, and isoelectric precipitation at pH 5.1 yields a protein extract which catalyzes the inactivation of a purified preparation of serum vasoconstrictor.

The authors wish to acknowledge the valuable assistance of Mr. John Means, Miss Elizabeth Hunt, and Miss Martha Bender. This investigation was partly supported by a grant from the U. S. Public Health Service, Cardiovascular Study Section.

16561

Anticonvulsant Properties of 5,5-Phenyl Thienyl Hydantoin in Comparison with Dilantin and Mesantoin.*†‡

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Dilantin (Diphenylhydantoin, U.S.P.) has been successfully employed for a decade in the treatment of grand mal and psychomotor epilepsy. In the past 2 years, Mesantoin (3-methyl, 5,5-phenyl ethyl hydantoin) has been used for the same purposes.¹⁻⁴ Still another congener, 5,5-phenyl thienyl hydantoin (Lilly 00079), has recently been used clinically with encouraging results.⁵ A number of previous communications have dealt with

the ability of Dilantin, Mesantoin and other congeneric substituted hydantoin, some of which have had initial therapeutic trial, to protect against or modify experimental seizures.⁶⁻¹⁶ Comparable data on 5,5-phenyl

* This investigation was made under the auspices of a grant-in-aid of research from the United States Public Health Service, RG-155 (C).

† Sodium Dilantin was kindly supplied by Dr. Oliver Kamm, Parke, Davis & Co.; Mesantoin, by Mr. Harry Althouse, Sandoz Chemical Works, Inc.; sodium 5,5-phenyl thienyl hydantoin, by Dr. K. K. Chen, Eli Lilly and Co.; and Metrazol, by Dr. E. A. Bilhuber, Bilhuber-Knoll Corp.

‡ The authors wish to thank Drs. O. P. Heninger, E. Wiemers, and J. W. Nixon, Utah State Hospital, Provo, Utah, for assistance in the human electroshock studies.

¹ Lennox, W. G., *Am. J. Psychiat.*, 1946, **103**, 159.

² Kozol, H. L., *Am. J. Psychiat.*, 1946, **103**, 154.

³ Loscalzo, A. E., *J. A. M. A.*, 1947, **135**, 496.

⁴ Aird, R. B., *California Med.*, 1948, **68**, 141.

⁵ Peterman, M. G., *South. M. J.*, 1948, **41**, 62.

⁶ Merritt, H. H., and Putnam, T. J., *Arch. Neurol. and Psychiat.*, 1939, **39**, 1003.

⁷ Hemphill, R. E., and Walter, W. G., *Lancet*, 1941, **1**, 446.

⁸ Kalinowsky, L. B., and Kennedy, F., *J. Nerv. and Ment. Dis.*, 1943, **98**, 56.

⁹ Merritt, H. H., and Putnam, T. J., *Epilepsia*, 1945, **3**, 51.

¹⁰ Toman, J. E. P., Swinyard, E. A., and Goodman, L. S., *J. Neurophysiol.*, 1946, **9**, 231.

¹¹ Goodman, L. S., Toman, J. E. P., and Swinyard, E. A., *Am. J. Med.*, 1946, **1**, 213.

¹² Swinyard, E. A., and Goodman, L. S., *Federation Proc.*, 1946, **5**, 205.

¹³ Toman, J. E. P., Loewe, S., and Goodman, L. S., *Arch. Neurol. and Psychiat.*, 1947, **58**, 314.

¹⁴ Swinyard, E. A., *Federation Proc.*, 1947, **6**, 376.

¹⁵ Merritt, H. H., and Brenner, C., *Bull. New York Acad. Med.*, 1947, **23**, 292.

¹⁶ Fabing, H. D., and Hawkins, J. R., *Ohio State M. J.*, 1948, **44**, 257.

TABLE I.
Protective Indices of 3 Antiepileptic Hydantoins by 4 Different Assay Methods in Rats.

Drug	TD ₅₀ mg/kg	Protective indices*			
		Max. electroshock seizure pattern	Normal electroshock threshold	Hydration electroshock threshold	Metrazol seizure protection
Mesantoin	50 ± 3 (24)	11.1 ± 0.71 (43)	0.69 ± 0.07 (25)	1.42 ± 0.25 (16)	0.91 ± 0.08 (26)
5,5-phenyl thienyl hydantoin	75 ± 6.5 (25)	4.47 ± 0.42 (49)	0 (20)	0.53 ± 0.05 (28)	0 (29)
Dilantin	104 ± 8 (20)	2.36 ± 0.24 (22)	0 (22)	1.1 ± 0.09 (26)	0 (30)

* For end-points employed and calculation of protective indices, see text.
± S.E. Figures in parentheses, No. of rats.

thienyl hydantoin have not as yet been published. This report is therefore concerned with the assay of the anticonvulsant properties of 5,5-phenyl thienyl hydantoin in comparison with Dilantin and Mesantoin.

Methods. (A). *Animal Assay.* Adult albino rats of the Sprague-Dawley strain were employed. For assays based on electrically-induced convulsions, Spiegel corneal electrodes were used and electroshock was delivered by means of a 60-cycle A.C. apparatus designed by Dr. L. A. Woodbury; the current delivered is independent of the external resistance. Stimulus duration was 0.2 second. Maximal electroshock seizures were obtained with a current of 150 m.A., which is 5- or 6-fold that required for minimal (threshold) seizures. For the assay based on experimentally lowered electroshock threshold ("hydration" threshold),¹⁷ rats were injected intraperitoneally with isomolar glucose solution, 10 cc/100 g, and determinations of drug-modified thresholds made 4 hours later. Chemoshock seizures were induced by 70 mg/kg. Metrazol injected subcutaneously, a dose which elicits convulsions in 97% of rats (CD₉₇).

Dilantin and the thienyl homolog were administered subcutaneously in aqueous solutions of their sodium salts; Mesantoin was injected intraperitoneally in solution in propylene glycol. Single doses were injected and the tests conducted after an interval (60 to 300 minutes) previously established as

the time for peak effect for the particular drug and test. The occurrence of minimal neurological deficit (such as loss of placing responses, ataxia, etc.) was considered as evidence of toxicity, and the toxic dose for 50% of rats (TD₅₀) was calculated for each drug by probit analysis. The effective dose of each drug for 50% of rats (ED₅₀) was similarly calculated for each test. Protective indices could then be obtained (TD₅₀/ED₅₀) for purposes of comparison of drugs.

The details of the assay methods employed have been reported elsewhere,^{10,11,17-19} but the end-points employed may be briefly summarized, as follows:

(1). *Maximal Electroshock Test.* A characteristic tonic-clonic seizure which is constant in duration and pattern follows the application of supramaximal current in non-medicated animals. The ED₅₀ is based on the abolition of the extensor component of the tonic phase of the convulsion.

(2). *Normal Electroshock Threshold Test.* The least current necessary for a minimal seizure was determined for each rat, which then served as its own control. The ED₅₀ is based on a 20% increase in threshold.

(3). *"Hydration" Electroshock Threshold.* The ED₅₀ is based on a 50% increase in the threshold previously lowered by reduction in extracellular cation.

(4). *Metrazol Seizure.* The ED₅₀ is

¹⁷ Swinyard, E. A., Toman, J. E. P., and Goodman, L. S., *J. Neurophysiol.*, 1946, **9**, 47.

¹⁸ Goodman, L. S., Swinyard, E. A., and Toman, J. E. P., *Arch. Neurol. and Psychiat.*, 1946, **56**, 20.

¹⁹ Toman, J. E. P., and Goodman, L. S., *Assoc. Research Nerv. and Ment. Dis.*, 1946.

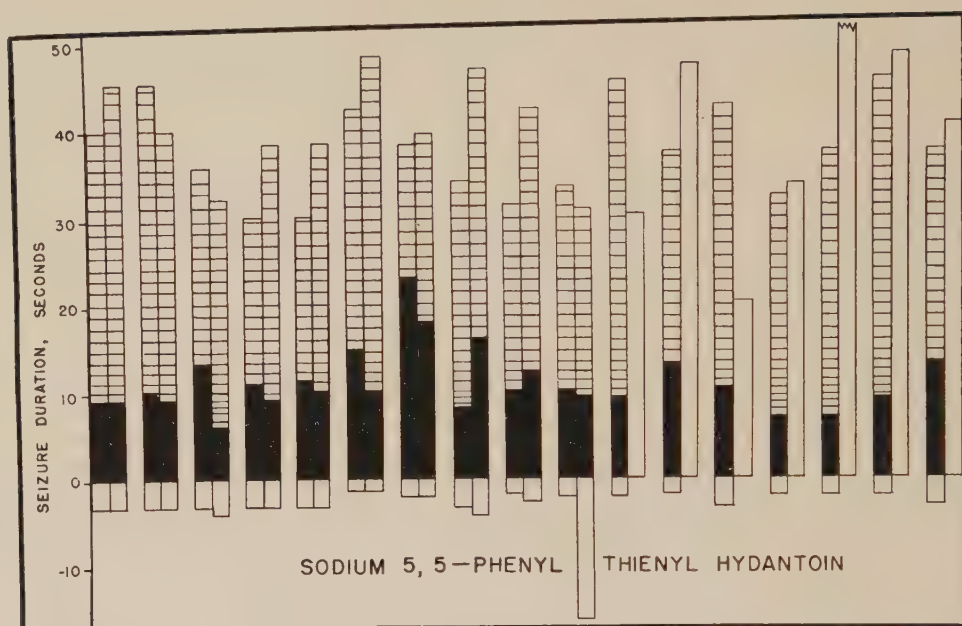


FIG. 1.

The Effect of 5,5-Phenyl Thienyl Hydantoin on Electroshock Seizure Pattern in Man.

Results of 17 separate electroshock tests on 10 non-epileptic patients. Each column represents a separate test, the left-hand column indicating the control seizure pattern and the right-hand column the seizure pattern after 3 days of oral medication with 5,5-phenyl thienyl hydantoin.

Open segment below the zero base-line indicates latency between current application and beginning of seizure; solid black segment, tonic component; horizontal-barred segment, clonic component; height of column, total duration; open columns, "missed shocks" (psychomotor seizures); serrated upper edge, indeterminate duration.

First 3 tests (starting at left of figure, first 3 pairs of columns), 0.13 g, twice daily; no change in seizure pattern. Next 2 tests, 0.13 g, 3 times daily; no change. Next 5 tests, 0.13 g, 6 times daily; no change. Next 3 tests, 0.13 g, 6 times daily; complete abolition of tonic-clonic seizures. Final 4 tests, 0.13 g, 9 times daily; complete abolition of tonic-clonic seizures.

based on complete protection against the CD_{97} of Metrazol.

(B). *Human Assay.* In addition to assays in rats, the ability of the 3 hydantoin to modify seizure patterns in non-epileptic patients undergoing electroshock therapy for psychiatric disorders was measured. The method was essentially a modification of the maximal electroshock test used in animals and has been fully described elsewhere.¹³ Control seizure patterns were compared with those obtained after 3 days of treatment with various dose levels of the hydantoins and several days after withdrawal of medication. Electroshock was delivered by a Rahm clinical apparatus through temporal electrodes, 120 to 150 volts, 0.2 to 0.4 sec. duration. Current intensity and stimulus duration were selected on the basis of previous experience

with each patient.

Results. The results of the animal experiments are shown in Table I. Mesantoin has the highest protective index by the maximal electroshock test, Dilantin the lowest and the thienyl compound is intermediate. In contrast to Mesantoin, neither Dilantin nor its thienyl homolog elevates the threshold for minimal electroshock seizures or affords protection against Metrazol-induced convulsions. All 3 compounds are capable of elevating the experimentally lowered (hydration) electroshock threshold; Mesantoin is the most effective by this test, Dilantin next and the thienyl derivative least.

All 3 hydantoins modify the pattern of electroshock seizures in man. As also occurs in animals, the tonic component of seizures is characteristically abolished. In-

deed, the tonic-clonic seizure may be entirely replaced by a "missed shock," especially when Mesantoin or the thienyl compound has been administered. Such "missed shocks" more nearly resemble psychomotor seizures.¹³ The doses of the 3 hydantoin necessary to modify electrically-induced seizures in non-epileptic subjects are in the upper range employed for the control of grand mal epilepsy. No toxic effects were noted from these doses. The results obtained with 5,5-phenyl thienyl hydantoin (Fig. 1) differ from those with Dilantin in that the convulsions were entirely replaced by psychomotor seizures and purely clonic convulsions were not observed. Mesantoin was intermediate in this respect.

Discussion. In general, 5,5-phenyl thienyl hydantoin resembles Dilantin in its anticonvulsant spectrum of activity in animals. Both compounds differ from Mesantoin in their inability to elevate threshold for minimal electroshock convulsions or to prevent Metrazol seizures. A striking antagonism exists between Metrazol and certain derivatives of oxazolidine 2,4-dione, such as Tridione and Paradione, which are effective in the petit mal triad; the relationship between this antagonism and drug efficacy in petit mal has been discussed.^{11,14,19} Inasmuch as the hydantoins at present employed clinically are generally ineffective against petit mal, the modest degree of Metrazol antagonism manifested by Mesantoin cannot as yet be related with its specificity of action in epilepsies. The methylated nitrogen and the ethyl radical on the carbon in position 5 independently bestow on Mesantoin the ability to antagonize Metrazol because both Nirvanol (5,5-phenyl ethyl hydantoin) and N-methyl diphenylhydantoin possess this action in slight measure.

On the basis of the experimental findings reported above, the prediction is advanced that 5,5-phenyl thienyl hydantoin will exhibit the same order of clinical usefulness as does Dilantin, that is, efficacy in grand mal and psychomotor epilepsy. The preliminary report of Peterman⁵ who employed the drug in 44 patients with epilepsy appears to

confirm such a prediction. However, this observer also recorded improvement in some cases of petit mal but made no mention of EEG diagnostic criteria. Although it is known that an occasional patient with pyknolepsy responds to hydantoin therapy, the results are usually not impressive and Tridione is the agent of choice. It will be interesting to observe whether other workers verify Peterman's results in petit mal.

Attention has already been directed to the advantages of multiple anti-convulsant assay tests, as employed here.^{10,11,19} No one test is sufficient to measure the potential antiepileptic potencies of a new drug, and a battery of indices permits a better comparative estimate. Both electrical and chemical methods should be included. Simple elevation of electrical threshold for minimal seizures is not adequate alone, as indicated by the inefficacy of Dilantin and 5,5-phenyl thienyl hydantoin by this test. However, both drugs elevate the experimentally reduced electroshock threshold, and markedly alter the pattern of maximal seizures in animals and man. It is believed at present that the maximal electroshock and Metrazol tests are most useful for preliminary screening of new drugs and that the other tests are best reserved for compounds exhibiting adequate indices by these two methods. The electroshock test in man also provides a means of preliminary screening for drug efficacy and toxicity. The many non-epileptic psychiatric patients receiving electroshock therapy provide adequate material for this assay, and the beneficial effect of the electroshock treatment is not compromised by a short period of antiepileptic drug administration.

Summary. A new anticonvulsant, 5,5-phenyl thienyl hydantoin, proposed for the treatment of epilepsy, has been compared with Dilantin and Mesantoin by 4 different assay methods in rats and examined for its ability to modify the electroshock seizure pattern in man. The thienyl congener more closely resembles Dilantin than Mesantoin in its spectrum of antiepileptic actions.

Factors Affecting the Survival of *Treponema pallidum* *in vitro*.*ROBERT A. NELSON, JR. AND HARRY G. STEINMAN.
(Introduced by Thomas B. Turner.)

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Preliminary quantitative experiments have been undertaken in an attempt to define various factors which influence the survival of *Treponema pallidum* *in vitro*.

Utilizing virulent *T. pallidum* (Nichols strain) obtained by Waring Blendor emulsification of rabbit testes in the early stage of acute syphilitic orchitis, it has been possible to compare the *in vitro* survival time of the organisms at a constant pH 1) in various gaseous atmospheres and 2) at varying temperatures.

Brewer's Fluid Thioglycollate Medium, used by one of us in previous work on the Reiter spirochete,¹ was arbitrarily chosen as a basal medium. This was supplemented by isotonic phosphate buffer and crystalline bovine albumin (Armour Laboratories) prepared as a 7% solution in isotonic saline pH adjusted to 7.0 with 1 M NaOH. Viability has been determined by counting 50 organisms at random under darkfield examination and recording the percent motile. To date, all specimens exhibiting motility have proven virulent as demonstrated by intracutaneous rabbit inoculation. There has been no in-

crease in the total number of organisms on prolonged incubation.

Since survival has repeatedly been limited to less than 24 hours at 37° C incubation, lower temperatures, 32° C and 27° C, have been utilized throughout this series of experiments.

It has been noted that survival of the organism is consistently prolonged when a 5% carbon dioxide, 95% nitrogen atmosphere is present over the culture tubes in the Brewer anaerobe jar. Half-life (*i.e.*, 50% of original number of motile organisms remaining motile) has been prolonged to 96 hours in specimens exposed to 5% CO₂ as compared to less than 24 hours of controls in 100% nitrogen or hydrogen. Specimens freely exposed to air had an even shorter half-life, *i.e.*, less than 12 hours.

Summary. 1. Preliminary observations on the survival of the virulent Nichols strain of *Treponema pallidum* *in vitro* indicate that under a given set of experimental conditions exposure to 5% CO₂ had a definite prolonging effect on survival time. 2. Survival was improved when temperatures below 37° C were used. 3. No indication of multiplication of the organisms has been noted. 4. Further quantitative experiments are in progress in an attempt to clarify the role of CO₂ and to define other physical and chemical factors which may affect this organism's survival *in vitro*.

* These studies were supported jointly by grants from the International Health Division of the Rockefeller Foundation and the Division of Research Grants and Fellowships of the National Institute of Health, United States Public Health Service.

¹ Eagle, H., and Steinman, H. G., *J. Bact.*, in press.

16563 P

Isolation of L Type Colonies from Typhoid Bacilli with the Aid of Penicillin.*

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It has been briefly reported that on blood agar plates inoculated with *H. influenzae* in the area surrounding penicillin cups colonies develop which in the appearance and in the morphology of the constituting organisms are closely similar to the L₁ developing in cultures of *Streptobacillus moniliformis*.¹ After this observation had been made, many bacterial species were studied on plates containing increasing concentrations of penicillin. L type cultures were isolated without difficulty on such plates from certain strains of *Bacteroides* and from a strain of *Flavobacterium*. Some of these strains produced similar colonies immediately after isolation without penicillin, but lost this property during prolonged cultivation.² Colonies of similar type were seen on penicillin plates inoculated with various other bacteria, but up to the present time, besides the species mentioned above, they have been isolated only from *Proteus* and typhoid bacilli. In this note the isolation of L type colonies from typhoid bacilli is described.

Two strains of typhoid bacilli were studied, one recently isolated from a carrier and an old laboratory strain of unknown origin. When the usual horse blood and ascitic agar plates containing 20 units of penicillin per cc or more were inoculated with these strains, no visible growth developed. Microscopic examination of the plates dis-

closed that some of the bacilli swelled up to large round bodies, and a few L type colonies started to develop. These remained very small and disappeared during further incubation. It was learned from experience with other bacteria that the composition of the medium exerts a great influence on the growth of these colonies. Plates of various composition were tested, and a macroscopically noticeable growth developed on nutrient agar diluted with an equal amount of broth to which 10% fresh horse serum, about 1/2% laked horse blood and 25 to 5000 units of penicillin per cc had been added. Colonies sometimes developed on aerobic plates, but more regularly under anaerobic conditions. They continued to increase in size during a week of incubation. The colonies were transferred to similar plates with a block of the agar cut out of the original culture. The transfer plates were incubated anaerobically. The colonies continued to grow on the agar block and grew into the fresh medium beneath it. A good growth of tiny colonies was obtained when the agar block was pushed on the surface of the medium after a few days and the plate was further incubated. In successive transfers the colonies grew faster and to a larger size regardless of whether or not the plates contained penicillin. Growth could be obtained aerobically and on the usual horse blood agar plates, but it remained slight and the culture died out after a few transfers. The appearance of the growth on the soft horse serum plates after the strain was well adapted to artificial cultivation is shown in Photograph I. Similar growth was obtained from both typhoid strains, but that obtained from the freshly isolated strain was more abundant and could be more easily maintained in cultivation.

This growth isolated from typhoid bacilli

* The expenses of this investigation have been defrayed in part by a grant from the Commonwealth Fund.

This is publication No. 102 of the Robert W. Lovett Memorial Foundation for the study of crippling disease.

¹ Dienes, L., PROC. SOC. EXP. BIOL. AND MED., 1947, **64**, 166.

² Dienes, L., and Smith, W. E., *J. Bacteriology*, 1944, **48**, 125.

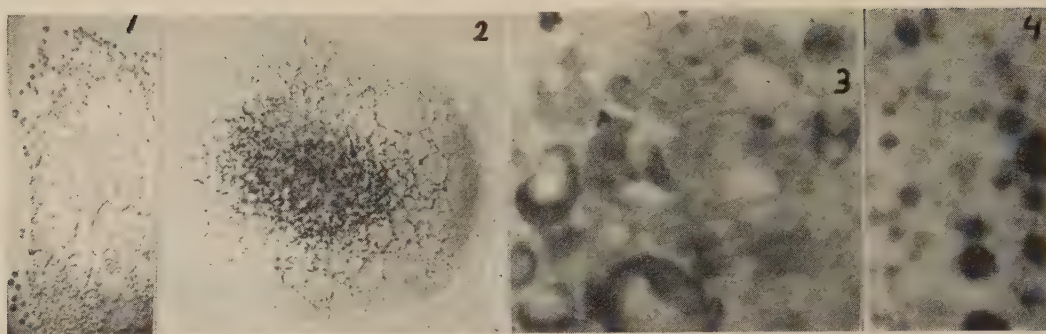


FIG. 1.

1. L type colonies from typhoid bacilli on soft horse serum plates. Slight magnification ($\times 2$). 2. One colony photographed unstained with the characteristic dense center and light periphery. ($\times 200$). 3. The periphery of a young colony consisting of vacuolated large bodies. ($\times 2000$). 4. Impression preparation from the dense center of a colony showing the small and the larger swollen forms. High magnification ($\times 3000$).

The photographs were made from cultures grown without penicillin.

differs in many respects from the usual bacilli. The young colonies consist of very small pleomorphic forms which grow into the medium and have a tendency to swell into round forms. First a dense colony embedded in the agar is formed. Later, on the surface of the colonies, the small forms swell to large round bodies and extend as a wide peripheral zone on the surface of the agar. The organisms are very soft and fragile, and the colonies often autolyze. The growth is slow and slight compared with the usual bacterial growth, and even high concentrations of penicillin exert no influence on it or on the morphology of the organisms. In all these properties the small colonies isolated from typhoid cultures are similar to the L_1 . The appearance of the colonies and the morphology of the organisms are so similar to that of the L_1 and the L type colonies isolated from *Bacteroides* that these cultures obtained from different species can hardly be distinguished on the basis of morphology alone. Their growth requirements and serological properties are different.

That the L type colonies originate from the typhoid bacilli and are not contamination in the cultures is indicated by various observations. These colonies were isolated from the cultures of 2 strains and were observed on all plates offering appropriate conditions. They developed only on plates inoculated with typhoid bacilli and not on others inoculated at the same time with various other species.

The L type colonies give a specific agglutination with typhoid sera. They originate from the bacilli in a manner similar to that of the L_1 growing from the large forms produced by penicillin. The detailed discussion of these observations must be left for future publications.

Summary. On soft horse serum agar containing high concentrations of penicillin, colonies corresponding to the L_1 of *Streptobacillus moniliformis* were isolated from typhoid bacilli. Cultures obtained from these colonies could be propagated indefinitely on similar media either with or without the addition of penicillin.

16564 P

Effect of Gonadal Hormones on Experimental Infection of Rats with *Brucella abortus*.*

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Ascoli¹ noted that there was a sex difference in the resistance of albino rats to intraperitoneal inoculations of *Brucella abortus*. Further studies involving over 1000 rats showed that the mature male rat is more resistant than the mature female and immature males and females to an intraperitoneal infection with a virulent strain of *Br. abortus*. This evidence suggested that androgen might account for the difference in resistance.

Several investigators have studied the effects of the estrogens on the course of experimental infections. In studies on tuberculosis, contradictory results have been obtained. Aycock and Foley² noted a decrease in resistance to tuberculosis in guinea pigs receiving α -estradiol dipropionate. Estrogenic substances were found to increase resistance in mice to *Diplococcus pneumoniae* Type I³ and to streptococcus infections.⁴ Testosterone propionate increased resistance in mice to *D. pneumoniae* Type I³ and to streptococcus infections.⁴ Progesterone had no effect on the resistance to pneumococcal infection in mice.³ *In vitro*, it was found that estrogens have a bactericidal action on Gram positive organisms, but have no effect on Gram negative organisms.⁵

Experimental. Albino rats from our colony were castrated at two age levels: sexually ma-

ture rats with an average weight of 150 g and sexually immature rats with an average weight of 80 g. The castration was performed 1 to 7 days before administration of the hormone. For each trial, the animals were divided into 3 groups: castrates receiving hormone, untreated castrates, and untreated intact animals, 5 or more animals were included in each group. In one trial estradiol dipropionate (Diovocylint[†]) was usually administered 2 days before infection by subcutaneous injection in oil at a dose level of 0.005 mg per rat. Mature males and females and immature females were used in this trial. In the other trial testosterone propionate (Perandren[†]) was administered by subcutaneous injections in oil at 2 dose levels; 0.5 mg per rat per day for 10 days (immature males and females) and 0.1 mg per rat per day for 10 days (mature males and immature males and females). Following the above treatments, the rats in all groups were injected intraperitoneally with approximately 4000 million *Br. abortus* organisms suspended in sterile saline. The number of survivors 5 days after infection was recorded.

The results given in Table I showed that all rats receiving testosterone propionate at both dose levels exhibited increased resistance to the infection. Of those rats receiving estradiol dipropionate, immature castrated females showed decreased resistance, and mature castrated females showed increased resistance to the infection. There was no significant difference in the group of mature males.

Another experiment was run on 15 immature intact males and 22 immature intact females. One half of each group was injected subcutaneously with 2.5 mg testosterone pro-

* Journal series paper, New Jersey Agricultural Experiment Station, Rutgers University, Department of Dairy Industry.

¹ Ascoli, A., PROC. SOC. EXP. BIOL. AND MED., 1947, **63**, 326.

² Aycock, A. L., and Foley, G. E., *J. Clin. Endocrinol.*, 1945, **5**, 337.

³ von Haam, E., and Rosenfeld, I., *J. Infect. Dis.*, 1942, **70**, 243.

⁴ Foley, G. E., and Aycock, W. L., *Endocrinol.*, 1940, **35**, 139.

⁵ Faulkner, G. H., *Lancet*, 1943, **2**, 38.

[†] Furnished by Ciba Pharmaceutical Products, Inc., through the courtesy of Dr. E. Oppenheimer.

TABLE I.
Influence of Gonadal Hormones on Resistance to *Brucella abortus* Infection in Rats.

Age level and sex	Intact Controls	Castrates				
		Controls	Estradiol dipropionate		Testosterone propionate	
			0.005 mg 2 days before infection	0.005 mg 7 days before infection	5 mg	1 mg
Immature males	1/5* 2/6	4/5 2/5			2/5	1/7
Immature females	3/8 3/6 3/5	0/6 2/3 5/11 2/8	3/8	8/11	0/8	0/5
Mature males	0/4	1/5 3/14	4/14			1/6
Mature females	5/6	6/13	2/13			

* Number deaths/total number rats.

TABLE II.
Results Obtained Using 2.5 mg Testosterone Propionate 6 Hours Before *Brucella abortus* Infection in Rats.

		No. rats	No. deaths	% mortality
Immature intact males	Treated	8	7	87.5
	Controls	7	7	100
Immature intact females	Treated	11	7	63.6
	Controls	11	11	100

pionate in oil 6 hours before infection. All rats were infected with 3800 million *Br. abortus* organisms via the intraperitoneal route. After 5 days, the mortality rate was as follows: males and females receiving no hormonal injection, 100%; males receiving testosterone propionate, 87.5%; females receiving testosterone propionate, 63.6%. (See Table II) These results indicate that the androgen increased the resistance of the females to the infection.

Discussion. It was thought that there might be some correlation between the effect of castration and subsequent hormone therapy on the size of the adrenal cortex and the resistance to infection. According to Dougherty *et al.*,⁶ the release of antibodies from the lymphocytes is controlled by adrenocortical hormone. Can it be assumed that, if the adrenal

cortex is larger than normal and contains more lipid, the animal is more resistant to infectious diseases? This might account for the fact that the female of the species in general is more resistant to infection than the male. In all species investigated the adrenal cortex of the female is larger than that of the male.⁷ In this experiment there was no correlation between the size of the adrenal cortex and the resistance to infection.

Summary. Testosterone propionate significantly increased the resistance to experimental *Br. abortus* infection in the small number of rats used in this experiment. The effect was more pronounced in the females than in the males. This correlates with the fact that A.L.D. for *Br. abortus* for mature male rats is twice that for mature females and immature male and female rats.

⁶ Dougherty, T. F., Chase, J. H., and White, A., *Proc. Soc. Exp. Biol. and Med.*, 1945, **58**, 135.

⁷ Giroud, A., and Santa, N., *C. R. Soc. Biol.*, 1940, **133**, 420.

16565

Isolation of Poliomyelitis Virus from Human Stools During the Incubation Period.*

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Since 1945, there have been 3 reports of the isolation of poliomyelitis virus from human stools collected during the incubation period. Pearson and Rendtorff¹ recorded the isolation of poliomyelitis virus from the stool of a child taken 11 days before the onset of an "abortive" case of the disease; Brown, Francis, and Pearson² from the stool of a boy taken 12 days before the onset of symptoms and 19 days before the onset of paralysis, and Gear and Mundel³ from the stool of a boy 12 days before onset of signs and paralysis.

Melnick⁴ has isolated virus from the stools of monkeys and chimpanzees several days prior to the onset of clinical signs and symptoms following subcutaneous and intracutaneous inoculation of these animals with poliomyelitis virus.

Poliomyelitis virus has been isolated by us from the stools of 3 more children, taken before the onset of the first signs and symptoms. Poliomyelitis in each of these children was what we believe to be the usual form of the disease, *i. e.*, without stiff neck, stiff back, or paralysis.⁵

In 1946, an outbreak of poliomyelitis occurred in a nursery school for children of

preschool age in Chicago. Among 29 children in the school, one case of paralytic and 2 cases of meningitic poliomyelitis occurred. Following the appearance of the paralytic case, one of us (A.E.C.) began close and careful observations on the rest of the children in the school. Daily clinical observations, including temperature determinations, were made on all of the children in the school and, whenever possible, specimens were collected for virus study. Nineteen of the remaining 26 children developed symptoms of poliomyelitis,⁵ but without paralysis, stiff neck or stiff back, and virus was isolated from the stools of 16 of these children collected at the onset of the first symptoms (fever, headache, drowsiness, myalgia, vomiting or constipation).

Stools collected prior to the appearance of symptoms in 4 of the 16 children with virus in their stools at the onset of symptoms, were available for testing.

Specimens were prepared for testing, and 2 monkeys were inoculated by the method previously described.⁶

The inoculated monkeys were considered to have been infected with poliomyelitis virus if, following inoculation, they developed fever, progressive paralysis, and microscopic lesions typical of poliomyelitis in the C.N.S. In one monkey no frank paralysis was observed, but it developed marked tremors of extremities and neck following high fever, and typical C.N.S. lesions were present. It was considered that this animal had been infected with poliomyelitis virus.

Table I shows the results of the stool tests described.

Poliomyelitis virus was isolated from the

* Aided by grants from the National Foundation for Infantile Paralysis, Inc., and its Cook County Chapter.

¹ Pearson, H. E., and Rendtorff, R. C., *Am. J. Hyg.*, 1945, **41**, 179.

² Brown, G. C., Francis, T., Jr., and Pearson, H. E., *J. A. M. A.*, 1945, **129**, 121.

³ Gear, J. H. S., and Mundel, B., *South Af. Med. J.*, 1946, **20**, 106.

⁴ Melnick, J. L., *J. Immunol.*, 1946, **53**, 277.

⁵ Casey, A. E., Fishbein, W. I., Gordon, F. B., Schabel, F. M., and Bundesen, H. N., *Transactions, Section on Pediatrics, American Medical Association, Chicago, June 23, 1948.*

⁶ Gordon, F. B., Schabel, F. M., Casey, A. E., and Fishbein, W. I., *J. Infect. Dis.*, 1948, **82**, 294.

TABLE I.
Poliomyelitis Virus in Stools Before Onset of Symptoms.

Name	CSA*	Date of onset and symptoms at onset	Dates of collection of stools tested	Virus isolations
El Bal	WF2½	8/5/46 Fever of 99.6° Axillary 1 day only	8/4/46 and 8/5/46 7/30/46 and 7/31/46 7/27/46	Virus isolated Virus not isolated Virus isolated 9 days before onset
Jo Jac	WF3	8/15/46 Fever 99.6° Axillary 1 day only	8/14/46 and 8/21/46 8/8/46 7/27/46	Virus isolated Virus isolated 7 days before onset Virus isolated 19 days before onset
Ri Sic	WM3½	8/7/46† Drowsiness and constipation	8/1/46, 8/5/46, and 8/8/46 7/26/46	Virus isolated 12 days before onset
Pa Woz	WF4	7/29/46 Fever	7/29/46 and 8/1/46 7/27/46	Virus isolated Virus not isolated

* Color, Sex, Age.

† This child had no stool on 7/27/46. Previous analysis of symptoms has indicated that constipation in contacts is of significance only if it occurs on successive days or, if on one day only, in conjunction with some other symptom typical of poliomyelitis (*i.e.*, fever, headache, myalgia, vomiting, or drowsiness).

stool of one child 19 days and 7 days before the onset of the clinical disease; from the stool of a second child 9 days before onset of the clinical disease, and from the stool of a third child 12 days before the onset of the clinical disease.

The onset dates (8/5, 8/7, and 8/15) in the 3 children with virus in stool prior to appearance of symptoms were the latest recorded among the 22 children in the nursery school who had the clinical disease. This probably indicates prolonged incubation

periods in these 3 children, since no symptoms or temperature rises were detected by earlier daily observations in the school beginning on July 25. The other children, including the one paralytic and the 2 meningitic cases, had onsets between July 14 and August 5.

These observations confirm those reported by other workers, namely, that the virus of poliomyelitis may be present in the stools of man for a considerable period of time prior to the onset of symptoms of the disease.

16566

Nature of the Refractory State Following Sublethal Dose of Human Placental Thromboplastin.*

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The toxicity of placental extracts upon intravenous injection into experimental animals has been studied repeatedly in the past half

century by investigators interested in the toxemias of late pregnancy. Schneider,¹ however, was the first to report on the refractory state in mice following sublethal injections of "placental toxin." This desensitization was temporary and lasted for ap-

* Aided by grants from the John and Mary Markle Foundation, New York City, and the James Fund of the University of California Medical School.

¹ Schneider, C., *Am. J. Physiol.*, 1946, **147**, 250.

proximately 8 to 12 hours. Animals became refractory almost immediately after intravenous injection of sublethal doses, and were then able to withstand 20 times the minimum lethal dose. Following the second injection, about 95% of the mice were found to have liver lesions consisting of widespread focal necrosis.² No explanation for the refractory state was offered other than that it "involved a change in some portion of the reacting mechanism itself."

Subsequently, Schneider³ identified the toxic principle of placental extracts as thromboplastin. This would suggest that the refractory state might be due to the exhaustion of one or more of the essential components of the coagulation system.

Materials and methods. Crude placental thromboplastin† was prepared by extracting a human placenta with cold 0.9% sodium chloride solution in a Waring blender at pH of 7.1. The mass was strained through cheese cloth, and the insoluble material reextracted in the same manner. The combined extracts were diluted with 5 volumes of cold water, and brought to a pH of 5.1 with 0.1 N. HCl. The precipitate, removed by centrifugation, was resuspended in 100 ml of distilled water, made isotonic with sodium chloride and adjusted to a pH of 7.4. The dry weight of the nondialyzable solids (protein) was 18.5 mg/ml of solution.

Commercial bovine thrombin (Upjohn) was dissolved in sterile saline to a strength of 200 units/ml. Bovine fibrinogen (Plasma Fraction I, Armour, 75% clottable) was prepared as a 2% solution in physiological saline.

Mice of either sex, weighing between 20 and 30 g, were used as test animals. All injections were given intravenously into the caudal veins, utilizing the technic of Nickson and Barkulis.⁴ Sublethal doses (LD₅₀) of

human placental thromboplastin (HPT) were calculated by interpolation on logarithmic-probit paper⁵ utilizing 6 animals at each dose level. Only those doses which killed less than 90 or more than 10% of each group were used in the calculation. From the best fitting line, the LD₅₀ was determined as well as the LD₉₉, i.e., that dose which would theoretically kill 99% of an infinitely large sample. Actually, this latter dose killed all control animals.

Results. A. Incoagulability of the blood during the refractory state. Following the injection of the sublethal dose (LD₅₀) 30 surviving mice were given the lethal dose and all survived, thus substantiating the existence of the refractory state. Nine of these animals showed liver lesions, similar to those described by Schneider, providing they were sacrificed within 48 hours. In an additional group, killed after one week, no lesions were observed. During the refractory state the blood would not clot *in vitro* even after the addition of thrombin or thromboplastin. This suggested that the desensitization was due to the exhaustion of fibrinogen.

B. Resistance to fatal doses of thrombin in the refractory state. Twelve animals, surviving the LD₅₀ dose of HPT were injected an hour later with a dose (50-60 units) of thrombin, an amount fatal to all control animals. None of this group showed any immediate observable effects, although one animal expired 12 hours later. While this experiment substantiated the fact that the fibrinogen was exhausted, it did not establish whether the prothrombin of the blood was likewise depleted.

C. Abolition of the refractory state by the restoration of fibrinogen. In order to test the thesis that the refractory state was due only to fibrinogen loss unaccompanied by prothrombin depletion, the refractory state was induced in 10 mice as before, and one hour later 6 mg of fibrinogen were injected intravenously. (This dose was calculated to restore the approximate normal fibrinogen content of the blood in a 20 g mouse.)

² Schneider, C., *Proc. Soc. Exp. Biol. and Med.*, 1946, **62**, 325.

³ Schneider, C., *Am. J. Physiol.*, 1947, **149**, 123.

† Human placental thromboplastin was generously supplied by Dr. F. F. Johnson of Cutter Laboratories, Berkeley, California.

⁴ Nickson, J., and Barkulis, S., *Science*, 1948, **107**, 229.

⁵ Miller, L., and Tainter, M., *Proc. Soc. Exp. Biol. and Med.*, 1944, **57**, 261.

An additional 30 minutes were allowed to elapse, and a lethal dose (LD₉₉) of the HPT proved immediately fatal to 8 of the 10 mice, with delayed death in the remaining 2. This indicates that prothrombin was still present, and substantiates the exhaustion of fibrinogen as the cause of the refractory state. The fact that the desensitization is lost gradually over a period of 8 to 12 hours is consistent with the known rate of regeneration of fibrinogen by the liver.

Conclusions. 1. Intravenous sublethal doses of human placental thromboplastin produce a refractory state in mice. During this state, the animals tolerate lethal doses of thrombin as well as thromboplastin, and the blood is incoagulable.

2. The refractory state is due to an exhaustion of the circulating fibrinogen. It may be abolished by restoring fibrinogen to the circulating blood, indicating that prothrombin is still present.

16567

Value of Soybean Trypsin Inhibitor in Preventing the Toxic Effects of Human Placental Thromboplastin.*

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As early as 1914, Gaifami¹ demonstrated that saline extracts of the human placenta, injected intravenously, are lethal to animals. Obata² investigated these effects extensively and showed, furthermore, that human serum neutralizes the toxic effects when mixed with the extract *in vitro*. Such lethal activity is not limited to placental tissue, for Krichesky and Mahler³ observed toxic effects from extracts of rabbit uteri, but only in the pregestational or gravid state.

Gizelt⁴ showed the presence of thromboplastin in the human placenta, and Chargaff⁵ has indicated by his recent quantitative studies that the thromboplastin content of the placenta is as high or higher than that of lung or brain. Schneider, in a series of

studies on the lethal effect of tissue extracts, finally concluded⁶ that the toxicity of placental extracts is entirely attributable to the thromboplastin content. He demonstrated that heparinization protects mice against such effects, and that the addition of heparin to the extracts *in vitro* likewise neutralizes the "placental toxin."

These facts have certain clinical implications pertaining to the origin of at least a part of the eclamptic syndrome in women. The circulatory arrangement of the human placenta is such that the maternal blood comes directly in contact with the trophoblastic epithelium, and the sequence of events which might arise as the result of a relative ischemia of the gravid uterus has been recently reviewed.⁷ In eclampsia, the liberation of placental thromboplastin into the maternal circulation might well account for the deposition of fibrin observed especially in the liver. Preliminary reports, furthermore, indicate that heparinization may be of value in the treatment of preeclampsia.^{8,9} There are, on the other hand, several disadvantages to

* Aided by grants from the John and Mary Markle Foundation, New York City, and the James Fund of the University of California Medical School.

¹ Gaifami, P., *Z. Biochem. u. Biophys.*, 1914, **17**, 74.

² Obata, I., *J. Immunol.*, 1919, **4**, 111.

³ Krichesky, B., and Mahler, J., *Endocrinology*, 1942, **30**, 616.

⁴ Gizelt, A., *Arch. ges. Physiol.*, 1913, **152**, 562.

⁵ Chargaff, E., *J. Biol. Chem.*, 1945, **161**, 389.

⁶ Schneider, C., *Am. J. Physiol.*, 1947, **149**, 123.

⁷ Page, E. W., *Obs. and Gyn. Survey*, in press.

TABLE I.
Protective Action of Soybean Trypsin Inhibitor.

STI (0.5 g/kg) administered	No. of mice	Mg (dry wt) of HPT (lethal dose)	Time (min.) between STI and HPT	Toxic effects					
				Immediate		Delayed		None	
				No.	%	No.	%	No.	%
None (controls)	28	0.8	—	28	100	0	0	0	0
Intravenously	24	0.8	30-45	2	8.3	1	4.2	21	88
Intramuscular	18	0.8	120-150	2	11	3	16.6	13	72
Subcutaneously	12	0.8	180+	6	50	6	50	0	0
I.V. (after mixing with HPT)	10	0.8	—	0	0	1	10	9	90

STI = Soybean Trypsin Inhibitor. HPT = Human Placental Thromboplastin. The differences between the immediate and delayed toxic effects of the HPT are explained in the text.

the clinical employment of heparin over long periods of time.

Macfarlane,¹⁰ utilizing a crystalline soybean trypsin inhibitor prepared by Kunitz,¹¹ showed that this protein inhibited the coagulation of blood by a "depression of the activity of thromboplastin." The purpose of the present study is to determine whether the soybean trypsin inhibitor would protect against the lethal effects of human placental extracts.

Materials and methods. The human placental thromboplastin (HPT) solution used† is the same as that described in the previous communication.¹² The dry weight of the dialyzed material (protein content) was 18.5 mg/ml.

The soybean trypsin inhibitor‡ (STI) is approximately 50% as pure as the crystalline material, and when assayed against fibrinolysin by the method of Loomis, *et al.*,¹³ contained 150 units/mg. A 6% solution was prepared in sterile 0.9% saline.

⁸ Maeck, J., and Zilliacus, H., *Am. J. Obstet. and Gynecol.*, 1948, **55**, 326.

⁹ Page, E. W., *Am. J. Med.*, 1948, **4**, 784.

¹⁰ Macfarlane, R., *J. Physiol.*, 1947, **106**, 104.

¹¹ Kunitz, M., *J. Gen. Physiol.*, 1947, **30**, 291 and 311.

† We are indebted to Dr. F. F. Johnson of Cutter Laboratories, Berkeley, California, for supplying the placental thromboplastin.

¹² Fulton, L., and Page, E. W., *PROC. SOC. EXP. BIOL. AND MED.*, 1948, **68**, 594.

‡ The soybean trypsin inhibitor was prepared and given to us by E. C. Loomis, Research Laboratories, Parke, Davis & Co., Detroit.

¹³ Loomis, E. C., George, C., Jr., and Ryder, A., *Arch. Biochem.*, 1947, **12**, 1.

Mice of either sex, weighing between 20 and 30 g, were used for the toxicity studies. All injections of the HPT were given intravenously in volumes not exceeding 0.3 ml. Estimations of the lethal dose (LD₅₀) were made as in the previous report.¹² The STI solutions were administered to mice by several routes, as indicated in Table I, and in addition were given intravenously to a guinea pig, rabbit and monkey. The *in vitro* tests were made by incubating the HPT and STI solutions together at 37° C for 30 minutes prior to injection.

Results A. Soybean trypsin inhibitor alone is not toxic. An amount of STI equivalent to 0.5 g/kg body weight was given intravenously to 30 unanesthetized mice without any observable evidence of immediate or delayed toxicity. To evaluate the sensitivity of other species, 0.5 g per kilo were given intravenously to a guinea pig and to a rabbit and 0.25 g/kg to a Rhesus monkey. There was no observable reaction, and when each of these 3 animals were reinjected with a smaller dose 2 weeks later, no anaphylactic response was noted. In the case of the monkey, blood samples drawn with silicone coated syringes and studied by the method of Jaques *et al.*¹⁴ showed a 4-fold increase in the plasma coagulation time a half hour after the initial injection of the STI.

B. Soybean trypsin inhibitor protects mice against the toxic effects of placental thromboplastin. The stock HPT solution was diluted with 3 volumes of saline in order to increase the accuracy of measurement.

¹⁴ Jaques, L., Fidler, E., Feested, E., and MacDonald, A., *Can. Med. Assn. J.*, 1946, **55**, 26.

Estimations of the lethal dose, based on extrapolated values obtained with logarithmic-probit paper, indicated that 0.3 ml (equivalent to 0.8 mg dry weight of protein) would theoretically kill 99% of animals. All 28 mice receiving this dose died within 3 minutes. The STI solutions were given intravenously to 24, intramuscularly to 18 and subcutaneously to 10 animals. The results are given in Table I. Two endpoints were observed; (1) immediate death, and (2) a delayed reaction consisting of convulsions, or dyspnea or death within the subsequent 24-hour period. The intravenous administration of the soybean material appeared to offer the greatest degree of protection, since 88% of the group showed no reactions of either type to lethal doses of HPT. The intramuscular route offered a moderate protection providing that two hours elapsed before giving the HPT. The subcutaneous route was far less effective. When the STI and HPT solutions were incubated *in vitro* before injection, there was

almost complete neutralization of the toxic effects.

In an attempt to determine the effective dosage of STI, the amount was reduced by half (*i.e.* from 18 mg to 9 mg) and given intravenously to an additional 12 mice. Two of the animals died within 5 minutes, and 9 additional mice showed delayed reactions. The lower dose level, therefore gives incomplete protection.

Conclusions. 1. When given intravenously to 4 species, the soybean trypsin inhibitor, in doses up to 0.5 g/kg, was devoid of any apparent toxic or anaphylactic effect. In the monkey, it was noted that the material caused a prolongation of the plasma coagulation time.

2. Given either intravenously or intramuscularly, the soybean trypsin inhibitor offered considerable protection against the subsequent administration of lethal doses of human placental thromboplastin, as shown in Table I.

16568

Inhibition of Cholinesterase by Adrenaline.

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Considerable evidence has accumulated which indicates that the neuro-humoral agents, acetylcholine and adrenaline, are not entirely antagonistic substances. Although the effector response to these two substances in most visceral structures is physiologically opposite, observations have suggested that in the central nervous system, in autonomic ganglia and at somatic motor nerve endings the one substance, adrenaline, may modify the action of the other and behave as a synergist.

Numerous experiments which have been reviewed by Burn¹ demonstrate the occurrence

of this phenomenon. Dale and Gaddum,² in working with denervated skeletal muscle, demonstrated that the effect of acetylcholine in causing contracture could be augmented by prior administration of adrenaline. von Euler and Gaddum³ and Bulbring and Burn⁴ have similarly demonstrated increased contracture in skeletal muscle by adrenaline.

In attempting to demonstrate that acetylcholine is responsible for the transmission of impulses in the spinal cord, Bulbring and

¹ Burn, J. H., *Physiol. Rev.*, 1945, **25**, 377.

² Dale, H. H., and Gaddum, J. H., *J. Physiol.*, 1930, **70**, 109.

³ v. Euler, U. S., and Gaddum, J. H., *J. Physiol.*, 1931, **73**, 54.

⁴ Bulbring, E., and Burn, J. H., *J. Physiol.*, 1936, **86**, 61.

⁵ Bulbring, E., and Burn, J. H., *J. Physiol.*, 1941, **100**, 337.

Burn⁵ also were unable to effect motor activity by perfusing the spinal cord with defibrinated blood containing acetylcholine unless adrenaline was also added to the perfusion fluid. This experiment and others demonstrate the potentiating action of adrenaline on acetylcholine in the spinal cord. More recently Stavraky⁶ reported that spinal neurones sensitized by partial isolation exhibit a period of marked excitation to adrenaline.

Of less direct evidence but highly suggestive of this same action are the general observations of increased reflex excitability in the intact animal under the influence of adrenaline and certain related sympathomimetic amines.

That adrenaline potentiates the action of acetylcholine by virtue of an inhibitory effect upon the enzyme, cholinesterase, appears likely. The experimental work reported below supports this possibility.

Experimental. Methods. The Warburg manometric technic was used employing double sidearm reaction flasks. Into the main compartment of each flask were placed definite quantities of enzyme preparation as noted below and bicarbonate-Ringer solution as prepared by Marnay and Nachmansohn.⁷ One-half cc of substrate (acetylcholine, to make a 0.003 molar solution) was added to a side arm of each series of flasks. Adrenaline hydrochloride was added in varying concentrations (see below) to the other side arms. The total fluid content of each flask was 3.0 cc. After gassing with a mixture of 5% CO₂-95% N₂ and equilibrating at 37.5° C, the contents of the side arms were tipped into the reaction chamber. Controls and blanks were run simultaneously. Gas evolution resulting from the liberation of acetic acid and its action on bicarbonate was measured in the conventional manner.

Two different lyophilized preparations of enzyme of a swine source were used, the one being extracted from parotid gland and the other from caudate nucleus. These were pre-

pared in part similar to the technic of Mendel and Mundell⁸ for the purification of a pseudo-cholinesterase from dog pancreas. They were extracted and fractionated with (NH₄)₂SO₄ at 0.48 and 0.85 saturations but purification by further precipitation, adsorption and elution was not carried out. The precipitates resulting from 0.85 saturation were mixed with water, dialyzed and centrifuged. The supernatant material was then lyophilized. Nitrogen determinations by a micro-Kjeldahl technic showed that the parotid preparation contains 14.85% nitrogen. On the basis of one mg of nitrogen being equivalent to 6.25 mg of protein this preparation is about 90% protein. In a reaction flask 0.875 mg of this preparation served to liberate approximately 220 cmm of CO₂ gas in 60 minutes, its activity per unit of dry weight being raised 5-fold over that of the original tissue.

The caudate nucleus obtained from hog brain was likewise extracted, fractionated and lyophilized. Nitrogen determinations showed that it consists of 14% nitrogen indicating that this preparation is also about 90% protein. Its activity was not increased over that of fresh material and in a reaction flask 2.7 mg dry weight of preparation liberated only 130 cmm of CO₂ gas in 60 minutes. As pointed out by Mendel and Rudney⁹ tissue from the central nervous system does not lend itself readily to purification.

Substrate specificity determinations give evidence that the former is a "pseudo" or "non-specific" cholinesterase since it hydrolyzes acetylcholine, benzoylcholine and several non-choline esters but does not hydrolyze acetyl-beta-methylcholine. The latter corresponds to the "specific" type of esterase. It does not hydrolyze non-choline esters, such as methyl butyrate, tripropionin, tributyrin and tricapyrin, nor does it hydrolyze benzoylcholine. It does split acetylcholine and acetyl-beta-methylcholine.

Adrenaline base was used, its solution being affected by the equivalent addition of acid to

⁸ Mendel, B., and Mundell, D. B., *Biochem. J.*, 1943, **37**, 64.

⁹ Mendel, B., and Rudney, H., *Biochem. J.*, 1943, **37**, 59.

⁶ Stavraky, G. W., *Am. J. Physiol.*, 1947, **150**, 37.

⁷ Marnay, A., and Nachmansohn, D., *J. Physiol.*, 1937, **89**, 359.

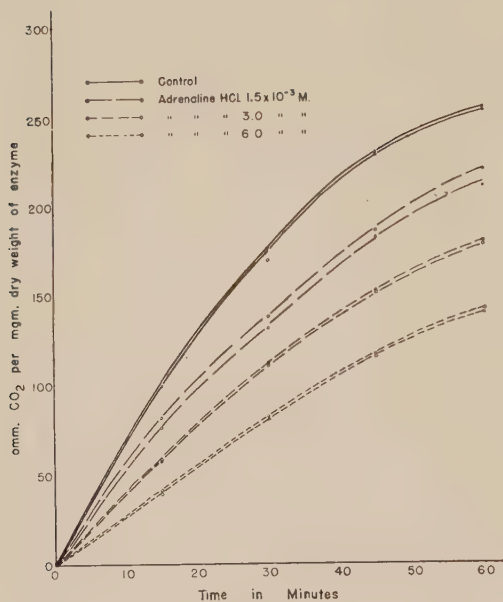


FIG. 1.

The inhibition of "non-specific" cholinesterase by varying concentrations of adrenaline HCl.

form the hydrochloride salt. Immediately after transferring it to the side arms of the Warburg flasks, the flasks and manometers were gassed to prevent its oxidation. In the experiments reported, the addition of adren-

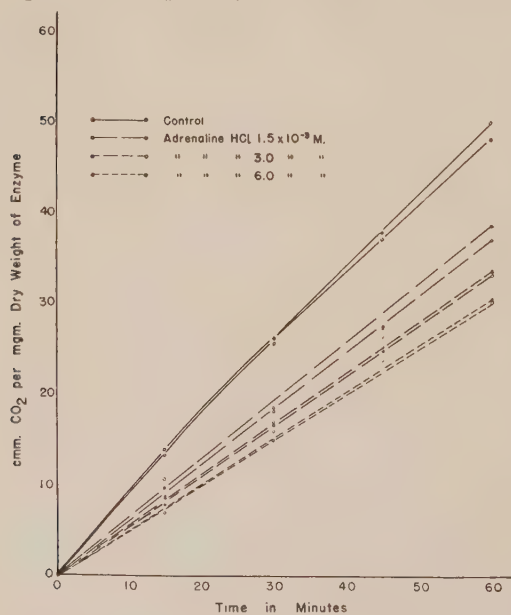


FIG. 2.

The inhibition of "specific" cholinesterase by varying concentrations of adrenaline HCl.

aline to the reaction chamber preceded the addition of the substrate.

Results. The results of the experiment are presented in the accompanying figures. It may be seen that adrenaline inhibits the ability of cholinesterase to hydrolyze acetylcholine. The inhibition of the enzyme is immediate, commencing with the addition of adrenaline. This observation was made in instances where the addition of adrenaline to the enzyme did not precede that of the substrate. In the case of the "non-specific" esterase (Fig. 1) it may be noted that at the end of 30 minutes the inhibition is 22.2, 35.8 and 53.2% with respective 0.0015, 0.003 and 0.006 molar concentrations of adrenaline. The inhibition by 0.003 molar adrenaline approximates that produced by ephedrine in the same concentration and by eserine in a 10⁻⁷ molar concentration.

In the case of the "specific" esterase the adrenaline inhibition at the end of 30 minutes is 29.1, 35 and 40.3% with the increasing concentrations of adrenaline (see Fig. 2).

A comparison of the extent of hydrolysis at the end of 60 minutes shows that in the case of the "non-specific" enzyme the inhibition for respective increasing concentrations of adrenaline is 15.2, 29.1 and 44.4%. In the case of "specific" enzyme it is 22.9, 31.8 and 38.2%.

Only one enzyme preparation of each type has been used. The experiments with the "non-specific" enzyme have been repeated and additional determinations were made with the "specific" type of enzyme.

To eliminate the possibility of the inhibitory effect being due to the addition of the "acid" solution of adrenaline the pH of the reaction was followed in another series of flasks using a 0.003M adrenaline solution. From Table I it may be seen that at the end of 30 minutes the pH of the flask mixtures containing the added adrenaline is not less than the pH of the mixtures not containing adrenaline. At the end of 60 minutes the pH is only very slightly less in the flask containing adrenaline and substrate than in the one not containing adrenaline. It

TABLE I.
pH of Reaction Mixtures.

	30 min.	60 min.
Buffer, enzyme, substrate	7.08	6.96
Buffer, enzyme, substrate, adrenaline	7.08	6.93
Buffer, enzyme, adrenaline	7.30	7.23

should be noted that the change in pH from the 30 to the 60 minute periods is not associated directly with either the adrenaline or the substrate because it occurred in all mixtures regardless of whether or not these were present.

Discussion. The potentiating action of adrenaline on acetylcholine has been attributed to various mechanisms of action. In his review on the relation of adrenaline to acetylcholine in the nervous system, Burn¹ suggests the possibility of an anticholinesterase action of adrenaline. He also suggests, however, that the potentiating effect may be explained by altered permeability of cell membranes. Torda and Wolff¹⁰ have demonstrated that adrenaline increases the aerobic synthesis of acetylcholine. These workers, indeed, have further suggested that both an increase in synthesis of acetylcholine and an inhibition of cholinesterase may cause certain cholinergic-like manifestations which are observable following stimulation of the sympathetic nervous system.

The anticholinesterase action of adrenaline has been reported previously, but for the most part, these reports have been of indirect evidence or confined to what is now recognized as "non-specific" enzyme. The experiments reported here elaborate upon this and in addition demonstrate a similar type of activity for the "specific" type of enzyme.

¹⁰ Torda, C., and Wolff, H. G., *PROC. SOC. EXP. BIOL. AND MED.*, 1944, **56**, 86.

This is of particular import inasmuch as it offers a mechanism for the potentiating effect of adrenaline on acetylcholine acting on the central nervous system and on preganglionic autonomic and somatic motor nerve fibers and endings.

Although the concentration of adrenaline used in this experiment is several thousand times that estimated to exist in the blood and tissues of the body the concentration of the other agents is also increased. A demonstration of parallel activity with physiological concentration would add support to the premises made.

In recognition of the finding by Torda and Wolff¹⁰ that adrenaline increases the synthesis of acetylcholine, a twofold mechanism of action may be ascribed to adrenaline in eliciting its synergistic response. Not only is a greater quantity of acetylcholine produced, but that which is produced is protected from hydrolytic cleavage and remains to exert, within limits, a greater physiological response.

Summary. 1. Lyophilized preparations of cholinesterase, identified as "specific" and "non-specific" in type, were fractionated from swine caudate nucleus and parotid gland, respectively.

2. Adrenaline inhibits the ability of these enzymes to hydrolyze acetylcholine.

3. The potentiation of acetylcholine activity by adrenaline is correlated with the inhibitory effect of adrenaline on cholinesterase.

The author wishes to thank Dr. L. A. Sweet of the Research Laboratories of Parke, Davis and Company for the adrenaline base used in these studies and Dr. Erick Baer of the Banting Institute, University of Toronto, for the benzoylcholine used in the substrate specificity determinations of the enzymes.

The Cardiodynamics of Tricuspid Insufficiency.*

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The purely physical effects of tricuspid insufficiency are often masked in patients by compensatory mechanisms as well as by the effects of concurrent cardiovascular disease.¹ While a few experimental observations of the dynamic changes produced by tricuspid regurgitation have been made,² little consideration has been given to the quantitative changes of pressure in the right auricle and large veins. Accordingly one phase of this study was undertaken to elucidate the immediate hemodynamic effects of uncomplicated lesions.

In order for the circulation to be maintained the output of the two ventricles must be equal. However, if a portion of the systolic ejection of the right ventricle is lost by regurgitation some mechanism must act promptly either to increase the tidal volume of the right ventricle or to decrease the systolic discharge of the left heart. The basic method by which the heart is able to maintain this equilibrium before extracardiac compensatory factors can come into action has not been fully worked out. Therefore, the second phase of this study was to determine the cardiac factors involved in effecting equivalent outputs of the two ventricles in the presence of an insufficient tricuspid valve.

Methods. Mongrel dogs were anesthetized

with morphine and chloralose (ca 75 mg per kilo) or morphine and sodium barbital (ca 200 mg per kilo). The chest was opened by a sternal splitting procedure and the fifth or sixth rib removed. Mild but adequate artificial respiration was maintained which was interrupted when records were taken. In order to maintain an adequate venous return most animals received warm saline by slow intravenous drip into the femoral vein during the experimental period. The right auricle was cannulated through the external jugular vein and the pulmonary artery via a side branch. Anatomically the left pulmonary artery was found to be more accessible and was generally used. Right ventricular pressures were registered by means of a blunt 15 gauge needle introduced through the ventricular wall. Aortic pressure was recorded by a sound introduced through the carotid artery to the arch of the aorta. All cannulae were securely clamped to avoid vibrations.

Gregg type manometers were used in an optical system similar to that described by Alexander and Webb.³ Valve lesions were produced by means of a trochar described by Wiggers.⁴ This was thrust through the right ventricular wall and introduced through the tricuspid valves. By withdrawing the plunger an insufficiency could be produced, while replacing it would permit the valves to function normally by closing tightly about the instrument.

Following each record a short calibration record was made with a fixed standard pressure. The zero pressure in all experiments was the hydrostatic level of the animal board. A complete calibration was made at the end of each experiment.

* Condensed report of results presented as a thesis in partial fulfillment of requirements for the degree of M.S. in the Graduate School of Western Reserve University.

† Supported by a grant from the Life Insurance Medical Research Fund.

¹ Bloomfield, R. A., Lauson, H. D., Cournand, A., Breed, E. S., and Richards, D. W., Jr., *J. Clin. Invest.*, 1946, **25**, 639.

² Rosenbach, O., *Arch. f. exp. Path. u. Pharm.*, 1878, **9**, 1; Rhil, J., *Berl. klin. Wochenschr.*, 1907, **44**, 825; MacCallum, W. C., *Johns Hopkins Hosp. Bull.*, 1911, **17**, 251; Wiggers, C. J., *Arch. Int. Med.*, 1915, **15**, 77.

³ Alexander, R. S., and Webb, E. A., *Am. J. Physiol.*, 1947, **150**, 272.

⁴ Wiggers, C. J., *Arch. Int. Med.*, 1915, **16**, 132.

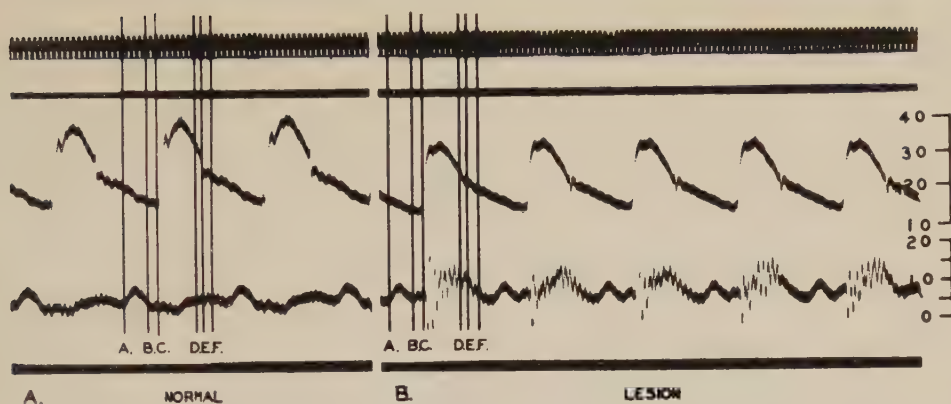


FIG. 1.

Record A shows the normal pulmonary (upper) and right auricular (lower) pressure curves. Record B shows the effects of tricuspid insufficiency. Time 0.02 second. Letters referred to in text.

Results and Interpretation. Records from 11 dogs were suitable for analysis. During tricuspid insufficiency the right auricular pressure rises at a steep slope during the systolic ejection phase of ventricular contraction (C-D, Fig 1), reaching a peak just before the second sound (E). A series of coarse vibrations are superimposed upon the curve during this phase. This is undoubtedly the murmur produced by the insufficiency. In all but one experiment of this study the auricular pressure began to fall during the phase of isometric relaxation (E-F). Wiggers and Feil⁵ found in mitral insufficiency that the left auricular pressure continued to rise until the end of ventricular systole. The cause of this difference between the right and left heart remains speculative. A possible explanation is that the volume of blood that regurgitates through an insufficient mitral valve must be accommodated in a comparatively small vascular bed, while that which backs up through the tricuspid valve can be dissipated toward the periphery by way of the large caeve. Consequently, it seems possible for auricular pressure to fall even though a slight regurgitation takes place during this period. It is conceivable that the early fall in auricular pressure may be prevented if the venous pressure is high and the peripheral transmission of the regurgi-

tated blood is impeded.

These studies confirm the observations of Wiggers and Feil⁵ that regurgitation does not occur through an insufficient A-V valve during isometric contraction.†

It has generally been accepted that tricuspid insufficiency *per se* causes a profound rise in right auricular and central venous pressures. That this is not necessarily the case is demonstrated in Fig. 1 and 2. Except during the systolic ejection and early diastolic phases of the cardiac cycle auricular pressure is not greatly elevated. The peak pressure reached during auricular systole is not changed as a result of the lesion. Apparently any elevation of mean venous pressure during uncomplicated lesions is due to the slight increase during ventricular ejection. The rise in venous pressure found in clinical cases perhaps is due to an increased blood volume or to mechanisms which restore arterial pressure and venous return to normal, or other concurrent lesions.

However, in all experiments the pressure in the auricle at the beginning of ventricular filling (F, Fig. 1) was always greater during the lesion than it was in the normal. Thus it is reasonable to assume that the rate of ventricular filling was increased.

The initial pressure in the right ventricle was measured by means of intraventricular

⁵ Wiggers, C. J., and Feil, H., *Heart*, 1922, 9, 149.

† Assuming the ventricle contracts isometrically when an insufficient valve exists.

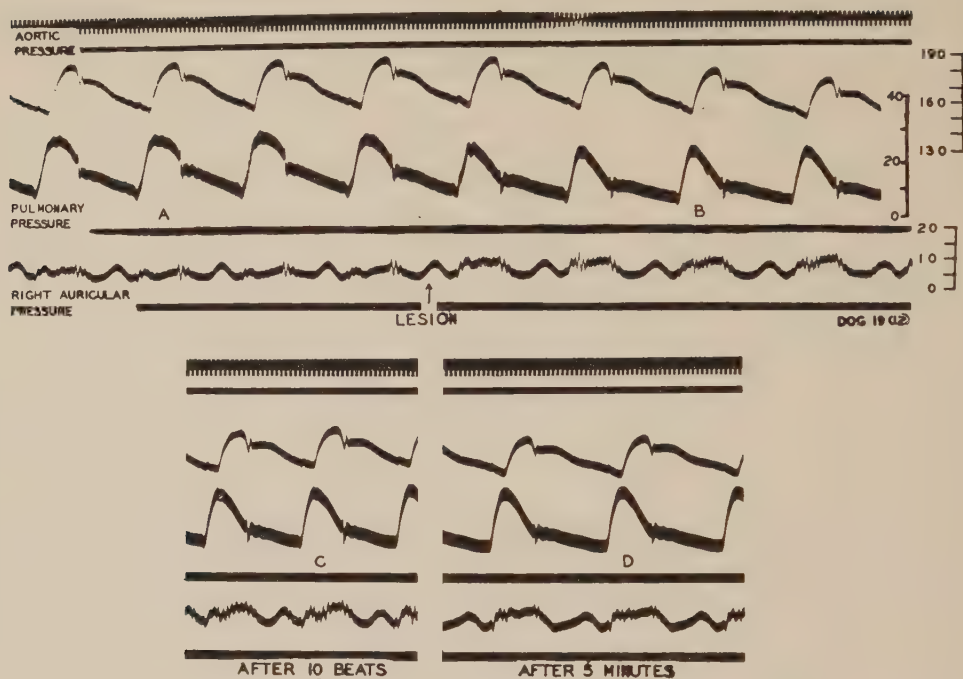


FIG. 2.

Sections of a record showing the immediate and subsequent effects of the lesion on aortic (upper), pulmonary (middle), and right auricular pressures (lower). Time 0.02 second.

pressure curves in 10 consecutive beats in 4 experiments both before and after the valvular lesion was produced. The initial tension increased in each case with an average rise of 2.3 mm of Hg, and a range between 1.7 and 2.4 mm. In spite of this increased initial tension with its accompanying dilation the amplitude of the ventricular pressure pulse was reduced due to the regurgitation.

Following tricuspid insufficiency the pulmonary pressure showed an immediate reduction in systolic, diastolic and pulse pressure. In seven experiments the pulse pressure fell an average of 2.7 mm of Hg with a range between 1 and 6 mm, while systolic pressure fell an average of 8.4 mm of Hg with a range of 5 to 10 mm.

The outstanding change in the form of the pulmonary pressure pulse is shown in Fig. 2. The smaller pulse pressure indicates a reduction in the effective systolic discharge of the right ventricle. The pulmonary pressure reaches its peak earlier and then falls rapidly to the incisura. This rapid drop from

the peak to the incisura shows clearly that right ventricular ejection of blood into the pulmonary system is not sustained during the latter part of systole as is normally the case.

As a result of this smaller effective systolic discharge by the right ventricle less blood is delivered to the left and its systolic discharge must decrease. Consequently, as shown in Fig. 2, aortic pulse pressure is reduced and systolic pressure falls as a direct result of the lesion.

The change in the filling and discharge of the right and left ventricles can be shown by means of theoretical volume curves. These are presented in Fig. 3. It will be noted that due to the increased filling pressure the right ventricle has a larger diastolic volume than the normal. As the duration of systole is not appreciably changed by the production of the lesion, the ejection of the ventricle is much more vigorous. The part of the ejection curve A-B represents the early phase of rapid ejection. During this phase the major part of

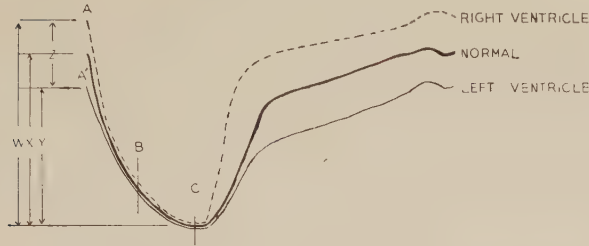


FIG. 3.

Theoretical ventricular volume curves showing the alterations in ejection and filling of right and left ventricles during tricuspid insufficiency. A and A', beginning of ventricular ejection; B, end of rapid ejection; C end of ejection. Further discussion in text.

the blood is ejected into the pulmonary system. The lower systolic pulmonary pressure and systolic collapse is explained by the dissipation of the systolic discharge back into the auricle. This regurgitated blood then causes more rapid and complete filling of the ventricle during diastole.

The left ventricle does not fill as completely as the right due to the lower pulmonary filling pressure; therefore, the diastolic volume A' is smaller. This, of course, will mean a smaller stroke volume ejected into the aorta.

In the theoretical volume curves presented in Fig. 3 W represents the amount of blood pumped by the right ventricle; X equals the output of the normal ventricle, while Y shows the volume of blood ejected by the left ventricle into the aorta. As both ventricles must move forward the same amount of blood in order to maintain the circulation, this must also represent the effective output of the right ventricle. By difference, then Z will show the volume of blood that is regurgitated back through the insufficient tricuspid valve.

Data presented refers to the immediate dynamic effects resulting from the tricuspid insufficiency. Ordinarily, compensatory mechanism such as changes in peripheral resistance operate to restore the circulation to normal. Therefore, it is not surprising that in clinical tricuspid disease the lesion, *per se*, may have little noticeable effect upon the

systemic circulation, and it is only after failure of these compensatory mechanisms that clinical signs and symptoms appear.

Summary. 1. The immediate cardiodynamic effects of uncomplicated tricuspid insufficiency were studied in dogs to elucidate the manner by which equilibrium between right and left ventricular output is maintained.

2. Pressure pulses were recorded from the right auricle, right ventricle, pulmonary artery and aorta by calibrated optical manometers.

3. As in mitral insufficiency, regurgitation occurs chiefly during ventricular ejection. However, except during late systole and early diastole, auricular pressure is not greatly elevated. The increased venous pressure found in clinical cases is apparently due to an increased blood volume or to the mechanisms associated with compensation or other cardiac lesions.

4. Comparison of pulmonary and aortic pressure pulses reveals that while the mechanism of left ventricular ejection is unchanged, that of the right is altered. The major portion of the stroke volume is ejected early in systole when regurgitation is less marked. The pulmonary pressure falls rapidly after reaching its peak, due to failing ventricular output later in systole when regurgitation becomes more pronounced. These changes are illustrated by theoretical volume curves of the two ventricles.

Resistance of Convalescent *Macaca mulatta* to Challenge with Homologous and Heterologous Strains of Poliomyelitis Virus.*

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(With the technical assistance of Robert George and Marilyn Thomas.)

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Reports concerning the immunity that occurs in monkeys following recovery from infection with poliomyelitis virus have been variable,¹⁻¹⁶ the incidence of such immunity in some instances being high and in others low; e.g., Flexner and Lewis¹ and Leiner and Von Wiesner² reported negative results while Burnet and McNamara³ and Flexner⁷ reported positive results. Often the same laboratory, e.g., Paul and Trask,⁵ Toomey⁸ and this laboratory^{12,15} reported both positive and negative findings. The amount of immunity

that develops would appear to depend upon several possible variables such as the strains of virus involved, the length of time between the attack and challenge, the challenge dose, and the route of inoculation. In general, a higher degree of immunity occurs when recovered animals have been challenged with an homologous strain of virus rather than with heterologous strains.

The current report was designed to determine, under as uniform and standardized conditions as possible, with an adequate number of monkeys, the comparative resistance of convalescent *Macaca mulatta* to challenge with both an homologous and with heterologous viruses.

Procedures. The BK strain which in 1941¹² in this laboratory had shown promise of producing a high degree of immunity to both homologous and heterologous challenge was selected as the base strain for this comparison.

1. **Preparation of Pool.** Twenty monkeys were inoculated with BK cord suspension from the seventeenth passage. Twelve developed symptoms within the first two weeks and from these the cord and brain stems were harvested during the acute phase of the disease. A pool was made by grinding the entire collection, suspending in saline at 10% concentration by weight, and centrifuging to remove the coarser particles. The supernatant was then placed in phials containing 5-25 cc each and stored in a deep freeze box where they were kept at a maximum temperature of minus 50°C until used.

2. **Titration of Pool.** This pool was titrated in 10-fold dilutions with the following results:

10-1	10-2	10-3	10-4	10-5
10/10	10/10	7/10	6/10	0/4
		PD ₅₀ 10-4		

* Aided by a grant from the National Foundation for Infantile Paralysis. Received for publication May 28, 1948.

¹ Flexner, S., and Lewis, P. A., *J. Exp. Med.*, 1910, **12**, 227.

² Leiner, C., and Von Wiesner, R., *Wien. klin. Woch.*, 1910, **23**, 817.

³ Burnet, F. M., and MacNamara, J., *Brit. J. Exp. Path.*, 1931, **12**, 57.

⁴ Schultz, E. W., Gebhardt, L. P., and Bullock, L. T., *J. Immunology*, 1931, **21**, 171.

⁵ Paul, J. R., and Trask, J. D., *J. Exp. Med.*, 1933, **58**, 513.

⁶ Jungeblut, C. W., *J. Inf. Dis.*, 1936, **58**, 150.

⁷ Flexner, S., *J. Exp. Med.*, 1937, **65**, 497.

⁸ Toomey, J. A., *J. Immunology*, 1938, **35**, 1.

⁹ Toomey, J. A., *Am. J. Dis. Child.*, 1939, **58**, 41.

¹⁰ Burnet, F. M., and Keigh, E. V., *M. J. Australia*, 1938, **2**, 130.

¹¹ Lennette, E. H., and Gordon, F. B., *J. Bact.*, 1940, **39**, 64.

¹² Kessel, J. F., and Stimpert, F. D., *J. Immunology*, 1941, **40**, 61.

¹³ Howe, H. A., and Bodian, David, *J. Exp. Med.*, 1941, **74**, 145.

¹⁴ Howe, H. A., and Bodian, David, *The Commonwealth Fund*, Oxford Univ. Press, 1942.

¹⁵ Moore, F. J., and Kessel, J. F., *Am. J. Hyg.*, 1943, **38**, 323.

¹⁶ Moore, F. J., Kessel, J. F., Hoyt, A., and Fisher, E., *Am. J. Hyg.*, 1942, **36**, 247.

3. *Selection of Dosage to Insure High Attack Rate.* Since all animals inoculated at 10^{-2} developed symptoms and 7 out of 10 at 10^{-3} , we selected an intermediate dosage, i.e., 1:500 dilution or 20 M.I.D. for the initial infection experiment.

4. *Standardized Inoculation Procedure.* One cc of inoculum containing the selected number of M.I.D. of virus was inoculated intracerebrally into the left pre-motor area.

5. *Daily Temperatures.* Rectal temperatures were recorded daily beginning the day following inoculation and being continued either until the acute phase of the disease was passed or for 30 days in non-attack animals.

6. *Spinal Fluid Cell Count.* A cell count was made on the spinal fluid of each animal prior to inoculation and again at the time of onset of symptoms or in non-paralyzed animals between the tenth and fourteenth day.

7. *Technic of Daily Muscle Checks.* Muscle weakness or paralysis was recorded daily in 4 degrees of severity, 1 representing the least amount of weakness and 4 the greatest degree.

8. *Homologous Challenge.* Reinoculation with homologous virus of 65 recovered animals 2 to 3½ months after first inoculation. (Points 3-7 above were repeated on animals at the time of reinoculation tests).

9. *Heterologous Challenge.* Reinoculation of 60 BK immune animals with 6 heterologous strains of virus one to 2 months following homologous challenge. (Points 3 to 7 also repeated).

Results. Attack Rate. One hundred and one animals were injected by the intracerebral route in the left pre-motor area with 1 ml dose of cord suspension containing 20 M.I.D. of BK virus. Ninety-five of these animals developed visible symptoms, the attack rate therefore being about 95%. Apart from natural deaths and the control animals sacrificed during the experiment, 50 of the animals recovered sufficiently to be used in the following test.

Challenge of Recovered Animals with Homologous Virus. Fifteen monkeys recovered from the titration experiment under Point 2, and the 50 recovered from the above

attack rate study, were allowed to convalesce for periods ranging from 2 to 3½ months and then subsequently were inoculated, some with 100 M.I.D. and some with 1000 M.I.D. of BK virus. Two of the 65 animals, monkeys 4837 and 5038, developed visible increased symptoms, motion picture records being kept of both. These animals were sacrificed and attempts made to transmit the virus to other monkeys. One of the attempts, with the cord of monkey 4837, was successful. Monkey 4837 gave the following history:-

First inoculation: February 14, 1947, intracerebral inoculation, with 1.0 cc of BK pool No. 3, 1-1000 dilution, i.e. 10 M.I.D. of virus.

First Symptoms:- February 21/47, right leg and left leg weak.

Maximum Paralysis, first attack right arm 2, left arm 2, right leg 4, left leg 4.

Residual Paralysis, June 4/47, right arm 0, left arm 1, right leg 4, left leg 4.

Challenge Inoculation:- June 4/47, with 100 M.I.D. of homologous BK virus from Pool 3.

June 18/48, Temperature 104° , also 70 cells in spinal fluid.

Maximum paralysis, second attack, right arm 2, left arm 3. Animal photographed and sacrificed on June 23/48.

Sections were made for histologic study. The pathologists reported changes compatible with poliomyelitis. Cord material was inoculated into monkeys 5076 and 5077, the latter developing characteristic paralytic symptoms on the tenth day.

From these observations it is projected that at least one recovered animal, in the series of 65 monkeys, upon challenge with homologous BK virus, developed a second attack. Sixty-three of these 65, however, definitely were resistant and it is concluded that they were immune to the challenge dose of virus used.

Challenge with Heterologous Virus. Sixty of the 63 monkeys definitely resistant to homologous challenge were divided into 6 groups of 10 each and each group subsequently challenged with a heterologous virus, 10% suspension of infected monkey cord, the fol-

TABLE I.

Virus strains in challenge	BK	McK	Ca	Fr	MV	Le	La
Recovered from BK virus	63/65	10/10	10/10	9/10	7/10	0/10	0/10

lowing 6 strains being used: McK and Le from the Los Angeles area isolated in our own laboratory; Ca from Dr. H. K. Faber in San Francisco; Fr from Dr. David Bodian in Baltimore; M. V., the much used strain from the Rockefeller Institute, procured by us from Dr. E. W. Schultz of Stanford University; and La, the Lansing strain received from Dr. W. McD. Hammon of the University of California. Table I gives the summary of the results of both homologous and heterologous challenge, the denominator representing the number challenged in each group and the numerator the number definitely immune in each group.

These results indicate a close antigenic relationship between BK, McK, Ca and Fr strains, partial relationship between BK and MV and no close relationship between BK and Le or La strains. It should be pointed out, however, that 7 of the La-challenged group and 5 of the Le-challenged group recovered from the second attack. Such a recovery rate is much higher than we have observed in this laboratory in animals that develop first attacks with La and Le viruses, the death rate from these two strains being near 100%. A partial immunity of BK recovered animals to these two strains therefore was apparent.

Sections from the cords of the second attack animals challenged with heterologous virus exhibited histopathologic changes compatible with poliomyelitis. Virus was recovered from one each of the MV, Le and La second-attack animals from which attempts were made to transmit virus.

Studies are now in progress to confirm the

antigenic relationship observed in this report by the additional methods of (a) challenging vaccinated animals with both homologous and heterologous viruses and (b) by testing the sera of the immune animals for neutralizing antibodies against both homologous and heterologous viruses.

Conclusions and summary. 1. Sixty-five *Macacca mulatta* recovered from a primary attack of poliomyelitis, induced by intracerebral inoculation of BK strain of virus, were subsequently challenged either with 100 or 1000 M.I.D. of the same pool of virus. Sixty-three exhibited complete resistance to challenge, only 2 showing increased paralysis following challenge. From one of these 2 animals, virus was recovered by passage 4 days following the onset of second attack symptoms which was 4 months following the first inoculation.

2. Sixty of the immune monkeys were divided into groups of 10 each and the monkeys of each group rechallenged with one of the following heterologous viruses, McK, Ca, Fr, MV, La and Le. All animals inoculated with McK and Ca viruses were immune and 9 of the 10 challenged with Fr were immune. These results indicate that BK, McK, Ca and Fr viruses are closely related and may be placed in a single immunogenic group. For the time being, we suggest that these and others which may be added to the group be designated as Group A or Group I of monkey-adapted strains from man.

3. The MV strain shows partial relationship since 7 of the 10 BK-recovered monkeys, challenged with MV, were immune.

4. The La and Le strains are not closely related to BK since all BK immune animals challenged with both of these two viruses developed second attacks. Whether these two belong to an identical group or whether each represents an independent strain must be determined by future study.

16571 P

Immune Response to Rabies Vaccine in Water-in-Oil Emulsion.

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The formation of neutralizing antibodies was compared in guinea pigs after the subcutaneous injection of rabies vaccine (rabbit brain containing inactivated rabies virus) given either in salt solution or in a water-in-oil emulsion with or without *Mycobacterium butyricum* (Table I). The technic of preparing the emulsions and other details of the method have been described.¹

Groups of 5 or 6 animals were given a single subcutaneous injection and bled 34 and 71 days later. The pooled sera were kept at -15° C until the neutralization tests were made. Equal volumes of serum-dilutions and infected rabies mouse brain suspensions were mixed, held at 37° C for 1 hour and at 4° C for another hour and injected into the brain of mice. The inoculum was 0.03 ml to which were added 20 Oxford Units of penicillin. The results are shown in Table II.

It is seen from this table that in the groups with vaccine in oil emulsions the neutralizing antibody titers were higher than with vaccine in saline solution. In another experiment using Vaccine B by the intraperitoneal route

the titers in the group with water-in-oil emulsion were at least 8 times higher than in the group with Vaccine B in salt solution. The killed mycobacteria did not seem to potentiate antibody production. In this connection it is interesting that *M. butyricum* and other mycobacteria have a potentiating effect on immunity responses to the influenza virus² and *S. typhosa*.³ Allergic encephalitis occurred only in the groups II, V, and VIII with *M. butyricum*. It may be emphasized here that single or few injections of brain in water-in-oil emulsion do not cause encephalitis unless mycobacteria or actinomycetes (*Nocardia asteroides*)⁴ are added to the emulsion.

Unpublished experiments on the formation of complement fixing antibodies against rabies virus are in harmony with the above results. They also show the potentiating effect of water-in-oil emulsion.

Titers of neutralizing antibodies in the blood are of significance with respect to protection against viral infection of the central nervous system. They are in a well-defined

TABLE I.
Material Injected.

Group	Vaccine A, ml	Vaccine B, ml	Vaccine C, ml	NaCl, 0.85%	Falva, ml	Paraffin oil, M. ml	Killed, <i>M. butyricum</i> , mg
I	0.8	—	—	1.2	—	—	—
II*	0.8	—	—	—	0.4	0.8	0.8
III	—	0.8	—	1.2	—	—	—
IV	—	0.8	—	—	0.4	0.8	—
V†	—	0.8	—	—	0.4	0.8	0.8
VI	—	—	0.8	1.2	—	—	—
VII	—	—	0.8	—	0.4	0.8	—
VIII†	—	—	0.8	—	0.4	0.8	0.8

* 3 of 5 animals died of allergic encephalitis before testing for antibodies.

† 2 of 5 animals died of allergic encephalitis before testing for antibodies.

1 Freund, J., Stern, E. R., and Pisani, T. M., *J. Immunol.*, 1947, **57**, 179.

2 Friedewald, W. F., *J. Exp. Med.*, 1944, **80**, 477.

3 Freund, J., Thomson, K. J., Hough, H. B., and Pisani, T. M., *J. Immunol.*, in press.

4 Freund, J., and Lipton, M. M., *Proc. Soc. Exp. Biol. and Med.*, 1948, **68**, 373.

TABLE II.
Neutralizing Antibody Titers of Sera of Guinea Pigs After a Single Injection of Rabies Vaccine.

Group	Serum dil.	1:5*	1:10	1:20	1:40	1:80	1:100	1:1000	1:10,000
34 Days—									
I Vaccine A			0/6†				6/6	6/6	6/6
II " A in oil + <i>M. butyricum</i>			0/5				1/5	5/5	6/6
III " B			1/6				4/4	6/6	6/6
IV " B in oil			0/5				0/6	0/6	6/6
V " B " " + <i>M. butyricum</i>			0/6				0/6	3/6	6/6
VI " C			0/6				3/5	6/6	4/5
VII " C in oil			0/5				0/6	2/6	4/5
VIII " C " " + <i>M. butyricum</i>			0/6				0/6	6/6	5/6
71 Days—									
III Vaccine B			2/7				8/8		
IV " B in oil			0/8				1/8	8/8	
VI " C		0/7	4/7	6/7	7/7	7/7	8/8		
VII " C in oil		1/7	0/8	0/7	1/7	6/7	7/8		

* 0.015 ml of a 1:2.5 dilution of serum + 0.015 ml of a fixed virus-brain suspension diluted 1:300,000.
Titer of virus suspension: 10-6: 8/8; 10-7: 5/8; 10-8: 3/8.

† 0 of 6 mice died of rabies.

numerical relationship to the antibody content of the CNS.⁵ Deviation from the ratio occurs when antibodies are produced in the CNS itself as a result of infection.⁶ Immunization with the aid of water-in-oil emulsion may be applicable to prophylactic immunization of dogs and to the production of "hyperimmune" sera for the treatment of man and lower animals after exposure to rabies infection. Habel⁷ found that greater protection

against rabies can be obtained in experimental animals with the combination of passive and active immunization than with the latter alone.

Summary. The incorporation of inactivated rabies vaccine into water-in-oil emulsion enhanced the antigenic property of the vaccine as judged by the formation of neutralizing antibodies. Allergic encephalitis did not occur unless mycobacteria were added to the emulsion.

⁵ Freund, J., *J. Exp. Med.*, 1930, **51**, 889.

⁶ Morgan, I. M., *Am. J. Hygiene*, 1947, **45**, 390.

⁷ Habel, K., *Pub. Health Rep.*, 1945, **60**, 545.

16572

Demonstration of Acid Phosphatase in *Endamoeba histolytica*.*

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Acid phosphatase activity can be demonstrated in *Endamoeba histolytica* using Gomori's histochemical method.¹ The amebae, originally isolated from human cases and grown in Balamuth's liquid media² or ob-

tained by rectal aspiration from an experimentally infected dog, consistently give strongly positive histochemical acid phosphatase reaction.

Although the method used is fundamentally the one described by Gomori, slight technical modifications are necessary to make it adap-

* Assisted by a Research Grant-in-aid, National Institute of Health, Bethesda, Md.

† Fellow, American Cancer Society.

¹ Gomori, G., *Arch. Path.*, 1941, **32**, 189.

² Balamuth, W., *Am. J. Clin. Path.*, 1946, **16**, 380.

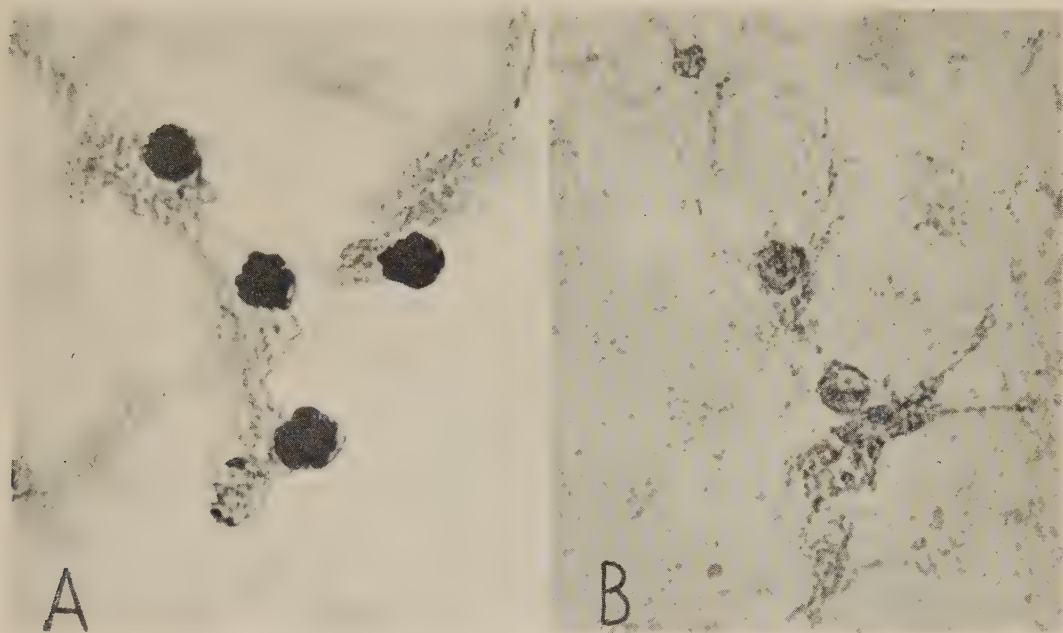


FIG. 1.

A. *E. histolytica* showing positive acid phosphatase reaction. Eosin counterstain. Acid phosphatase is indicated by black deposits in the organism. (Magnification $\times 510$.)

B. Control. *E. histolytica* showing no black deposits. Eosin counterstain. The granularity seen in the background and in the organisms is red in the original side. (Magnification $\times 510$.)

table for amebae. Recently, Rabinovitch, Junqueira and Mendes,³ and Bayliss, Glick and Siem,⁴ have reported modifications of Gomori's method for bone-marrow smears and bacteria and fungi respectively. In dealing with *E. histolytica* care must be taken not to let the smears dry completely before immersing in the fixative, since the limiting membrane of the ameba is very fragile and ruptures upon drying. The smears should be allowed to stand at room temperature until they become "glossy" and are then fixed in chilled acetone for 30 seconds following Gomori's technic, although positive phosphatase reactions have been obtained by the present workers in smears that have been fixed in acetone in the refrigerator for as long as 26 hours. Incubation at room temperature (26-29° C) has been found to be satisfactory,

therefore eliminating the need for a water bath. The period required to produce a positive reaction with *E. histolytica* has been found to be at least 45 minutes at room temperature. The amount of precipitate seen in the amebae increases with increased incubation. At 2 hours the cytoplasm is almost completely filled with precipitate, while incubation overnight brings out even greater amounts of precipitate. Control preparations were made in all experiments, either by omitting the glycerophosphate from the buffered reagent or by adding to the control substrate sodium fluoride (M/300), a known acid-phosphatase inhibitor. Since controls were negative, positive results can be interpreted as being due to phosphatase activity. Fig. 1 shows positive reaction (left) and control (right).

Summary. The presence of acid phosphatase in *E. histolytica* has been demonstrated by a modification of Gomori's histochemical method.

³ Rabinovitch, M., Junqueira, L. C. U., and Mendes, F. T., *Science*, 1948, **107**, 322.

⁴ Bayliss, M., Glick, D., and Siem, R. A., *J. Bact.*, 1948, **55**, 307.

Occurrence of Rabies Virus in the Blood of Developing Chick Embryo.

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The question whether rabies virus is present in the blood of infected animals has been a case of contention for several decades.^{1,2} Pasteur and his associates³ elicited rabies in a dog by intravenous inoculation of blood from a rabies infected rabbit. Marie,⁴ reporting his results, reviewed the work of Magendie, Renault and Galtier, and of Paul Bert, who were unable to produce rabies in dogs with inoculations or even transfusions of blood from infected animals. Marie⁴ tested more than 20 samples of blood obtained from experimentally rabies-infected animals but obtained only two positive responses. Marie and Urbain⁵ were able to demonstrate rabies virus in the blood of 25% of rabbits infected intracerebrally provided that solutions of powdered milk, gonacrin or tuberculin also were injected into the brain tissues of the animals.

Konradi⁶ observed that rabies could be transmitted by pregnant dogs to the fetus and offspring, and believed that this was an indication of the hematological spread of the virus. In view of the results he obtained in his experiments with various animals, Konradi suggested that the ability to produce rabies by inoculation of blood from infected animals depended on the degree of susceptibility of the test-animals and claimed that guinea pigs were superior to dogs or rabbits

in experimental rabies work. However, Ohira⁷ in attempting to repeat Konradi's blood transmission experiments in a much larger group of animals, succeeded only in one of a series of 9 experiments in eliciting rabies in a rabbit, but not in guinea pigs. Schweinburg and Windholz,⁸ in a series of 18 experiments, inoculated parabiotic rats intramuscularly with rabies virus but in spite of the common blood stream, only the injected animals developed rabies whereas the parabionts showed no sign of illness, nor could the presence of the virus in the brain tissues of the latter be demonstrated by subinoculation into guinea pigs.

Presumptive evidence was reported by Remlinger and Bailly⁹ who fed dog ticks (*Rhipicephalus sanguineus*) on 2 dogs, one of which had been inoculated intracerebrally with fixed virus and the other with street virus. Later the presence of rabies virus was demonstrated in the ticks. Neujean¹⁰ observed Negri bodies in cultures consisting of a mixture of blood from infected animals and a commercial preparation called Liquoid which had been kept at 25° C for 3 to 4 days.

In the course of studies on rabies infection in the developing chick embryo,¹¹ it became apparent that rabies virus is distributed throughout the tissues of the embryo with no

¹ Koch, J., Gustav Fischer, Urban und Schwarzenberg, Berlin-Wien, 1930, 656.

² Schweinburg, F., *Erg. Hyg. Bakt. Imm. u. Exp. Therap.*, 1937, **20**, 6, 8, 10, 45.

³ Pasteur, L., Chamberland, C. E., and Roux, P. P. E., *C. R. Acad. Sci.*, Paris, 1884, **98**, 457.

⁴ Marie, A. C., *C. R. Soc. Biol.*, Paris, 1905, **58**, 544.

⁵ Marie, A. C., and Urbain, A., *C. R. Soc. Biol.*, Paris, 1931, **106**, 166.

⁶ Konradi, D., *Centralbl. Bakt. Parasit. u. Infektionskrk.*, 1908, **47**, I Abt. Orig., 203.

⁷ Ohira, T., *Centralbl. Bakt. Parasit. u. Infektionskrk.*, 1920, **84**, I Abt. Orig., 528.

⁸ Schweinburg, F., and Windholz, F., *Virchow's Arch. Path. Anat. Phys. u. Klin. Med.*, 1930, **278**, 23.

⁹ Remlinger, P., and Bailly, J., *Ann. Inst. Pasteur*, Paris, 1939, **62**, 463.

¹⁰ Neujean, C., *Rec. Trav. Sci. Med. Congo Belge*, 1945, No. 4, 193 (as abstracted in *Trop. Dis. Bull.*, 1946, **43**, 189.)

¹¹ Koprowski, H., and Cox, H. R., presented at the 47th General Meeting of the Society of American Bacteriologists, Philadelphia, May, 1947, *J. Bact.*, 1947, **54**, 74, abstract.

particular affinity to the central nervous system. These results suggested a hematological spread of the virus and, therefore, presence of the virus in the blood of the embryo. Data obtained in experiments dealing with the latter possibility are presented in this paper.

Material and methods. The Flury¹² strain of rabies virus, obtained through the courtesy of Dr. Harald N. Johnson of the Laboratories of the International Health Division of The Rockefeller Foundation, was used throughout the experiments. Fertile hens' eggs, incubated for 7 days, were inoculated into the yolk sac with 0.25 ml of a 10% suspension of embryos infected with the 23rd egg-passage virus. Following inoculation the eggs were held at 36° C for the duration of the experiment and the embryos were bled from the artery as follows.* The egg shell, first washed with a 3% tincture of iodine solution, is cracked open with forceps. A probe is inserted under the exposed artery which is then separated from the adjacent tissue and flooded with sterile saline solution. A 27 gauge, one-half inch long needle, attached to a 1 ml tuberculin syringe, is inserted into the lumen and the blood slowly withdrawn. Blood specimens collected from individual embryos, without use of any anticoagulant, were pooled until an amount sufficient for experimental purposes was obtained. By sedimentation at 1,000 r.p.m. for 5 minutes, the pooled blood was separated into red blood cells (RBC) and plasma. The packed RBC were resuspended in a small volume of physiological salt solution and centrifuged. The supernate was discarded and the sedimented RBC were resuspended in saline solution to a volume equal to the original blood pool. Six albino Swiss mice, 21 to 28 days old, were inoculated intracerebrally with the RBC

suspension, whereas similar groups of mice received the undiluted and serial tenfold dilutions of the chick-embryo plasma. Ten per cent rabbit serum-saline was used as diluent.

Experimental. Three series of experiments (A, B and C) were performed. In experiment A the chick embryos were bled on the 1st, 4th and 5th day after inoculation. In Experiment B starting on the 3rd day after inoculation, and in Experiment C on the 6th day, the embryos were bled each day throughout the incubation period. The results of the experiments are summarized in Table I.

The number of embryos from which the blood was pooled varied in each experiment, depending on the volume of blood obtained from individual embryos. If one may judge by the results obtained in a single experiment, no virus was recovered from the blood pool on the first day after inoculation. Great difficulties were encountered in obtaining blood from embryos under 10 days of age and, therefore, one only may adduce that virus was present in the blood pool from 43 embryos bled on the 3rd day after inoculation. The virus was present in the blood from that day until death of the embryos which, in the case of embryos inoculated when 7 days old, usually occurred immediately before or at the time of hatching. The virus was recovered from both the embryo-plasma and from RBC, although in one instance, (Experiment A, 5th day after inoculation) the RBC suspension failed to elicit signs of illness in the injected mice.

Because of the degree of experimental error involved in this type of experiment, it is not possible to state with certainty the day on which maximal concentration of virus was present. In Experiment B the highest LD₅₀ titer was obtained with blood collected from embryos on the 9th day after inoculation, but a sudden drop in titer occurred with blood drawn on the 11th day. However, for reasons stated above, these results may be more apparent than real. In Experiment C the highest LD₅₀ titer was obtained with embryo-plasma secured on the 13th day after

¹² Leach, C. N., and Johnson, H. N., *Am. J. Trop. Med.*, 1940, **20**, 335.

* The authors are indebted to Mr. C. Alexander and Dr. D. H. Moore, of the College of Physicians and Surgeons at Columbia University, for the demonstration of their technic in bleeding chick embryos.

TABLE I.
Titrations of Chick Embryo Blood Pools for Circulating Rabies Virus.

Exper.	No. of embryos in pool	Embryos bled Days after inoc.	Mortality ratio of mice inoculated intracerebrally with:						
			Embryo-RBC*	Embryo-Plasma					
			Undiluted	Undil.	10-1	10-2	10-3	10-4	LD ₅₀ titer
A	22	1	0/6	0/5	0/5				
B	43	3	5/5	6/6					
A	14	4	1/6	5/6	2/6				
B	14	4	1/6	6/6					
A	8	5	0/6	1/5	0/6				
B	10	5	6/6	3/6	1/6				
C	7	6	6/6	6/6	6/6				
B	10	7	6/6	6/6	6/6	4/6	1/6	0/6	10-2.35
C	4	7	6/6	6/6	6/6	6/6	1/6	0/5	10-2.60
B	9	8	6/6	6/6	6/6				
C	4	8	6/6	6/6	6/6	6/6	0/6	1/6	10-2.60
B	7	9	5/5	6/6	6/6	6/6	4/5	0/6	10-3.40
C	4	9	6/6	6/6	6/6	0/6	1/6	0/6	10-1.60
B	7	10	6/6	6/6	6/6	6/6	1/6	0/6	10-2.60
C	4	10	6/6	6/6	6/6	2/5	1/5	1/5	10-2.20
B	4	11	5/5	6/6	0/6	0/6	0/6		10-0.50
C	4	11	6/6	6/6	6/6	2/6	0/6	0/6	10-1.75
B	4	12	6/6	6/6	4/6	3/6	0/6	0/6	10-1.70
C	8	12	6/6	6/6	2/6	2/5	2/5	0/6	10-1.40
B	7	13	6/6	6/6	6/6	1/6	1/6	0/6	10-1.70
C	4	13	5/5	6/6	6/6	4/5	3/6	0/6	10-2.85
B	6	14	6/6	6/6	6/6	6/6	0/6	0/6	10-2.50
C	8	14	5/5	5/5	4/4	1/6	0/6	0/4	10-1.60
B	7	15	6/6	6/6	6/6	0/6	0/6	0/6	10-1.50
C	6	15	6/6	6/6	4/6	1/5	0/6	0/6	10-1.35

* RBC—Red blood cells.

inoculation. However, the difference between this and the titers obtained with blood pools drawn on the 7th and 8th days after inoculation falls well within the limits of experimental error. Thus, it may be assumed, that the concentration of rabies virus in the blood of infected embryos was fairly constant, the daily fluctuations being of minor importance.

Summary and comments. The Flury strain of rabies virus was inoculated by the yolk-sac route into 7-day-old chick embryos.

By means of intracerebral injections into mice, the virus was recovered from the chick-embryo blood secured from the 3rd to the 15th post inoculation day. The similar results obtained in two series of experiments seem to exclude the possibility of a major experimental error. While these experiments do not prove or disprove the infectiousness of mammalian blood from rabies infected animals, they do indicate a completely different mechanism of dissemination of the virus in embryonated eggs.

16574

Reverse Interference Between Simian and Murine Poliomyelitis Virus in Guinea Pigs.*

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Earlier work has shown that certain strains of mouse-adapted human poliomyelitis virus (Col SK, MM) which have lost the greater part of their monkey pathogenicity will effectively interfere with the propagation of simian virus in rhesus monkeys. Murine infection in mice, on the other hand, could not be significantly influenced by the administration of monkey poliomyelitis virus. Nor was it possible to inhibit regularly the growth of murine virus in minced embryonic mouse brain tissue culture by adding monkey poliomyelitis virus to the medium.¹ Subsequent attempts to arrest the propagation of murine virus (Col SK) in embryonated eggs by previous or simultaneous implantation with simian virus (Aycock) have likewise failed to give unequivocal results.²

The question as to whether this viral antagonism is strictly a unilateral reaction—as would seem from the above observations—or

whether interference may also be demonstrated in the reverse direction is of more than theoretical interest. In one case the phenomenon would be confined to whatever therapeutic significance it may possess, in the other, it might conceivably lend itself also to diagnostic application. Evidence that such reverse reaction may actually occur under favorable circumstances has already come from the work of Dalldorf and Whitney.³ These authors reported that hamsters following intracerebral injection with simian virus, or with filtrates of faeces collected from human cases of poliomyelitis, exhibited a well marked resistance towards subsequent intraperitoneal challenge with MM murine poliomyelitis virus.

Previous attempts to demonstrate reverse interference in mice having failed—possibly because of the extreme susceptibility of the albino mouse to murine infection—it was decided to investigate whether the phenomenon could be successfully elicited in a less sensitive animal, such as the guinea pig. Results obtained in a small series of experiments

* Aided by grants from the Philip Hanson Hiss, Jr., Memorial Fund and from anonymous donors.

¹ Jungeblut, C. W., and Sanders, M., *J. Exp. Med.*, 1942, **76**, 127; Jungeblut, C. W., *J. Exp. Med.*, 1945, **81**, 275.

² Jungeblut, C. W., unpublished data.

³ Dalldorf, G., and Whitney, E., *Science*, 1943, **98**, 477.

TABLE I.
Interference Between Simian and Murine Virus in Guinea Pigs.

Experiment	No. of guinea pigs	Blocking injection		Challenging injection		Route	Result	
		Simian virus	Dose	Murine virus	Dose		Paralysis	No paralysis
I	7	RMV	{ 0.1 cc i.c. 0.5 cc i.p.	MM	1 cc 10-1	i.p.	0	7
	5	Aycock	"	"	"	"	2	3
II	7	Control	"	"	"	"	4	3
	5	RMV	"	"	0.1 cc 10-1	i.c.	0	5
III	4	Aycock	"	"	"	"	0	4
	5	Control	"	"	"	"	2	3
IV	2	RMV	"	"	1 cc 10-1	i.p.	0	2
	4	Control	"	"	"	"	3	1
V	6	Aycock	"	"	"	"	0	6
	5	Control	"	"	"	"	2	3
VI	3	Aycock	"	"	0.1 cc 10-1	i.c.	1	4
	5	Control	"	"	"	"	2	1
VII	5	Aycock	"	"	"	"	2	3
	5	Control	"	"	"	"	5	0
Total (Exps. I-VII)	20	Simian	"	"	1 cc 10-1	i.p.	2	18
	16	Control	"	"	"	"	9	7
	24	Simian	"	"	0.1 cc 10-1	i.c.	4	20
	18	Control	"	"	"	"	11	7

are recorded in this communication.

Methods. Two different strains of simian passage virus, *i.e.* the Aycock strain and the RMV strain, were used for blocking injection. The challenging virus was the MM strain of mouse-adapted human poliomyelitis virus. The tests were carried out in young guinea pigs (175 g weight), which are moderately susceptible to infection with MM murine virus by either the intracerebral or intraperitoneal route. The interfering effect was measured by injecting the guinea pigs first with a large dose of 10% glycerinated simian virus suspension (*i.c.* and *i.p.*) and, after a uniform interval of 24 hours, challenging the prepared animals intracerebrally or intraperitoneally with approximately 10-100 ID₅₀ of freshly harvested murine virus. The interval between the two injections was advisedly held brief to exclude any concurrent effects of immunity. A suitable number of control guinea pigs, challenged with the same dose of MM virus following preparation with 10% glycerinated normal monkey cord suspension, accompanied each experiment. A total of 7 different experiments were run, which are presented in Table I.

It appears from Table I that in every one of the 7 experiments the incidence of paralysis following challenge with MM virus was considerably reduced in the animals prepared with simian virus as compared with the corresponding controls. Experiments I, III, IV, V, and VI were winter experiments with MM virus at the peak of its guinea pig pathogenicity. Experiments II and VI had been conducted in the summer months when murine virus undergoes some seasonal drop in virulence for guinea pigs.⁴ Combining the data of the several experimental series, it will be seen that of a total of 44 guinea pigs prepared with simian virus, which were challenged either intracerebrally (24) or intra-

peritoneally (20) with MM virus, 4 (16%) responded with paralysis to intracerebral challenge and 2 (10%) to intraperitoneal challenge. By contrast, of a total of 34 control animals which received MM virus intracerebrally (18) or intraperitoneally (16), 11 (61%) or 9 (56%), respectively, became paralyzed. In other words, in the prepared group the morbidity rate was approximately one-fourth to one-fifth that observed in the control group. These figures are in close agreement with those previously published by Dalldorf and Whitney,³ who reported that among a total of 36 prepared hamsters 9 (25%) became paralyzed upon intracerebral challenge with MM virus, whereas among 29 control hamsters, 28 (97%) developed paralysis.

Summary. The above results indicate that reverse interference between simian and murine virus occurs with about the same frequency in rodents as direct interference between murine and simian virus in monkeys. The phenomenon may therefore be said to be reciprocal in character. Adjustments in the quantities of both viruses or variations of the time interval may further improve the efficiency of the reaction. In judging these results it must be borne in mind that successful operation of the interference phenomenon depends upon a delicate balance between the ability of the interfering virus to occupy susceptible cell receptors and the invasive potency of the challenging virus. It is therefore, perhaps, not without importance that the two strains of simian virus employed successfully in this work were passage strains of unusual virulence. One experiment with simian virus of lesser virulence has given less clearcut results. Leaving alone the question of its specificity, it therefore seems doubtful whether this reaction can be used for diagnostic purposes to detect virus in human cases of the disease without resorting to some form of preliminary concentration of the pathological material (faeces, blood, nerve tissue, etc.).

⁴ Jungeblut, C. W., *PROC. SOC. EXP. BIOL. AND MED.*, 1945, **58**, 177.

Simplified Technique for Titrating Influenza Virus Neutralizing Antibody in the Chick Embryo.*

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Following the studies of Burnet¹ and Hirst,² the chick embryo has largely supplanted the mouse for estimating neutralizing antibodies for influenza virus. A method is described here which eliminates the necessity of harvesting fluid from each egg individually and testing it for virus.

The following strains of influenza virus were used in these tests and in preparation of rabbit sera: 2 classical influenza A strains (PR8 and Weiss), 2 strains of influenza A isolated during the outbreak of 1943 (MB and CP), 2 atypical strains of influenza A isolated early in 1947^{3,4} (FM and EB) and the Lee strain of influenza B. All had been passed repeatedly by the allantoic route in eggs. Both human and rabbit immune sera were tested. Each of the latter was obtained 2 weeks after a single intravenous injection of 0.1 ml of virus infected allantoic fluid. Acute and convalescent sera were obtained from patients with clinical influenza in 1947.⁴ The serum virus mixtures were prepared as described by Hirst.² The sera were heated at 60° C for 20 minutes; 4-fold dilutions were then made in saline; and a constant amount of virus (adjusted to contain 500-1000 allantoic 50% infecting doses in 0.1 cc of the final mixture) added to each of the serum dilutions and mixed thoroughly. The mixtures were then allowed to stand for a

few minutes at room temperature; 0.1 ml was inoculated into the allantoic sac of 10-day-old embryos and 0.25 ml, or about 50-100 LD₅₀, was injected into the yolk sac of 7-day embryos. From 4 to 6 embryos were inoculated with each dilution. The virus suspension in 20% horse serum broth was titrated by inoculating 2 series of eggs in a similar manner with 10-fold serial dilutions, one series allantoically and the other by the yolk sac. Eggs were incubated at 35° C and embryos dying during the first 24 hours discarded.

At the end of 48 hours, allantoic fluids from eggs inoculated into the allantoic sac were tested for presence of virus by addition of ½ ml of 1% hen cells to ½ ml of the fluid. The 50% serum protective titer was then calculated by the method of Reed and Muench.⁵ The yolk sac inoculated eggs were candled daily and the deaths recorded up to the fifth day after inoculation, when survivors were discarded. Tests of the yolk and/or allantoic fluids of dead eggs with hen cells revealed the presence of virus in the yolk in all instances and in the allantoic fluid in one-half of the eggs. Most of the survivors had virus demonstrable in the yolk but rarely in allantoic fluid. The 50% serum protective titer was then calculated.

Rabbit sera obtained before immunization and acute phase human sera showed titers of <1:4 in almost every instance. The comparative titers for neutralizing antibody obtained by the allantoic and yolk sac techniques on the immune human and rabbit sera with different viruses are given in Table I.

It is of interest to point out first that the nonimmune rabbit sera and the acute

* Aided by a grant from the National Foundation for Infantile Paralysis.

[†] Senior Fellow in Medical Sciences, National Research Council.

¹ Burnet, F. M., *Australian J. Exp. Biol. and Med. Sc.*, 1941, **19**, 39.

² Hirst, G. K., *J. Immunol.*, 1942, **45**, 285.

³ Smadel, J. E., *Bull. U. S. Army Med. Dept.*, 1947, **7**, 795.

⁴ Morgan, H. R., Barnes, M. W., and Finland, M., *J. Lab. & Clin. Med.*, 1948, in press.

⁵ Reed, L. J., and Muench, A., *Am. J. Hyg.*, 1938, **27**, 493.

TABLE I.

Comparison of Titers of Influenza Virus Neutralizing Antibodies in Human and Rabbit Immune Sera Using Allantoic and Yolk Sac Routes.

Rabbit serum	Neutralizing titer for influenza virus*					
	PR8†		Weiss†		MB†	
	Allantoic	Yolk sac	Allantoic	Yolk sac	Allantoic	Yolk sac
PR8	<4/2048	<4/>4096	<4/128	<4/810	—	—
Weiss	<4/8	<4/16	<4/128	<4/162	—	—
MB	<4/64	<4/64	<4/512	<4/1024	<4/2048	<4/>4096
CP	<4/512	<4/256	—	—	—	—
FM ₁	<4/<4	<4/<4	—	—	<4/<4	<4/<4
EB	<4/<4	<4/<4	—	—	<4/4	<4/<4
Lee	<4/<4	<4/<4	—	—	—	—

Human serum	Neutralizing titer for influenza virus*					
	PR8†		EB†		FM ₁ †	
	Allantoic	Yolk sac	Allantoic	Yolk sac	Allantoic	Yolk sac
EB	<4/32	<4/91	<4/6	<4/28	11/181	4/64
GP	—	—	<4/<4	<4/23	—	—
DW	—	—	<4/<4	<4/<4	—	—
HB	<4/512	<4/512	<4/512	6/>1024	<4/160	<4/425
PW	<4/<4	<4/<4	<4/<4	<4/<4	—	—

* Expressed as reciprocals. Numerator is pre-immunization or acute phase titer; denominator is immune or convalescent titer. Each pair of sera tested simultaneously by both methods.

† For allantoic tests the dose was approximately 500 ID₅₀ and for the yolk sac method about 50 LD₅₀.

—, not done.

phase human sera almost all failed to show neutralization by either the allantoic or yolk sac methods. This suggests that the presence of neutralizing antibody is a reliable index of recent antigenic exposure to influenza virus.

These data show that the presence of neutralizing antibodies for influenza viruses can be determined by the yolk sac technic and that this method has essentially the same range of sensitivity and specificity as the method employing the allantoic sac route of inoculation. The differences in the results obtained by the two methods is probably

within the range of experimental error. Application of this method in a study of antigenic differences among strains obtained in an outbreak of influenza are presented elsewhere.⁴

Summary and conclusions. A yolk sac technic for the titration of serum neutralizing antibody for influenza viruses has been described which eliminates the necessity of testing each individual egg for infectivity since death of the embryo is used as a measure of infection with the virus.

Influence of Anti-Metabolites of Essential Vitamins on Growth and Acid Production of *Lactobacillus acidophilus* (Hadley).*

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The findings of Hill and Kniesner¹ and of Weisberger and Johnson² indicate that pantothenic acid, nicotinic acid and thiamine are essential for the maximum growth and acid production of the oral *Lactobacillus acidophilus* recovered from the saliva of caries active patients. In addition to being necessary for the optimum growth and acid production of the oral lactobacilli, thiamine and nicotinic acid in the role of coenzymes take part in the degradation of glucose to lactic acid.³ The production of acids from fermentable foodstuffs about the sheltered areas of the teeth by the acidogenic and aciduric organisms of the oral cavity in concentrations sufficient to cause a decalcification of tooth substance is the basis of the widely accepted chemico-parasitic theory of the etiology of dental caries. Since it has been shown that pyridine-3-sulfonic acid, pantooyltaurine and pyrithiamine are metabolic antagonists‡ of nicotinic acid, pantothenic acid and thiamine

respectively for some species of bacteria and laboratory animals,^{5,6,7} this study was undertaken to determine whether these compounds exerted an inhibitory effect on the growth and acid production of the oral *Lactobacillus acidophilus*, the organism intimately associated with the caries process.

Materials and Methods. A pure culture of the *Lactobacillus acidophilus* (Hadley) re-

TABLE I.
Composition of Test Medium.*

Constituent	Quantity
Casein hydrolysate	2.5 ml
Salt solution A†	0.5 "
" " B‡	0.5 "
Glucose	1 g
Sodium acetate	600 mg
Asparagine	25 "
Tryptophane	10 "
Cystine	10 "
Guanine hydrochloride	500 µg
Adenine sulfate	500 "
Xanthine	500 "
Uracil	500 "
Pyridoxine hydrochloride	40 "
Riboflavin	20 "
Calcium pantothenate	20 "
Nicotinic acid	20 "
Thiamine hydrochloride	10 "
Folic acid	1 "
Biotin	0.5 "
Distilled water to 50 ml.	

* Double strength, Landy and Dicken medium adjusted to pH 6.8 with NaOH.

† Salt solution A:

K ₂ HPO ₄	5 g
KH ₂ PO ₄	5 g

Distilled water to 50 ml.

‡ Salt solution B:

MgSO ₄ · 7H ₂ O	10 g
NaCl	0.5 g
FeSO ₄ · 7H ₂ O	0.5 g
MnSO ₄ · 2H ₂ O	0.337 g
Distilled water to 250 ml.	

⁵ McIlwain, H., *Brit. J. Exp. Path.*, 1940, **21**, 136.

⁶ Snell, E. E., Chan, L., Spiridanoff, S., Way, E. L., and Leake, C. D., *Science*, 1943, **97**, 168.

⁷ Woolley, D. W., and White, A. G. C., *J. Exp. Med.*, 1943, **78**, 409.

* Northwestern University Studies in Nutrition at the Hillman Hospital, Birmingham, Ala.

† We wish to thank Dr. Hans Molitor of Merck and Company for providing us with the anti-metabolites used in these studies.

¹ Hill, T. J., and Kniesner, A. H., *J. D. Res.*, 1942, **21**, 467.

² Weisberger, D., and Johnson, F. G., *J. D. Res.*, 1946, **25**, 35.

³ Tauber, H., *Enzyme Chemistry*, John Wiley and Sons, Inc., New York, 1937.

‡ Metabolic antagonists or competitive inhibitors have been defined as "the mutually antagonistic components of a system in which the role of either the metabolite or the anti-metabolite is reversibly and competitively interfered with by its antagonist, the relative amounts of the two substances in combination with the enzymic or other cellular components being dependent upon their relative affinities for them and upon their concentration-ratio."⁴

⁴ Welch, Arnold D., *Phys. Rev.*, 1945, **25**, 687.

TABLE II.
Effect of Pantoyltaurine, Pyridine-3-Sulfonic Acid, and Pyrithiamine on Growth and Acid Production of the *Lactobacillus acidophilus* (Hadley)*.

Tube	Metabolite-anti-metabolite ratio	Growth (packed cell volume)†	% decrease in acid production‡
Pantoyltaurine (μg)			
1,000	1:250	+++	00.0
2,000	1:500	+++	24.2
4,000	1:1000	+++	43.3
7,500	1:1875	±	92.2
10,000	1:2500	—	100.0
Pyridine-3-Sulfonic Acid (μg)			
1,000	1:250	+++	00.0
2,000	1:500	+++	00.0
3,000	1:750	+++	00.0
4,000	1:1000	+++	27.7
5,000	1:1250	+++	35.9
7,500	1:1875	+++	55.9
10,000	1:2500	++	71.8
Pyrithiamine (μg)			
470	1:235	+++	00.0
1,880	1:940	+++	00.0
2,820	1:1410	+++	30.6
3,350	1:1675	+++	41.5
5,000	1:2500	++	73.7
6,500	1:3250	+	87.4

* 72-hr reading.

† 0.0 -0.1 cc, —

0.1 -0.25 cc, ±

0.25-0.5 cc, +

0.5 -1.0 cc, ++

1.0 -2.0 cc, +++

‡ Acid production in control tube = 100%.

covered from a carious lesion was obtained from the American Type Culture Collection (ATCC 4646) and used as the test organism in these studies. The special medium consisted of that described by Landy and Dicken.⁸ Ten cc, the amount present in each tube, contained 4 μg of nicotinic acid, 4 μg of calcium pantothenate, and 2 μg of thiamine hydrochloride (Table I). Pyridine-3-sulfonic acid, pyrithiamine and pantoyltaurine were added to 3 different series of tubes in amounts ranging from 1,000 through 10,000 μg, 470 through 6,500 μg, and 1,000 through 10,000 μg respectively. The tubes were autoclaved and inoculated with one drop of inoculum, the preparation of which has been described in detail in a previous publication.⁹ They were shaken and incubated at 37.5°C for 72 hours. Growth was determined by centrifuging the cultures in Hopkins vaccine tubes for 10

minutes at high speed and then immediately measuring the packed cell volume. Acid production was determined by titrating the centrifugates with 0.1N NaOH to the pH of the uninoculated control tubes. A Beckman pH meter was employed for this purpose.

Results. The effect of the antimetabolites on the growth and acid production of the test organism at the end of the experimental period is shown in Table II. Of the amounts used in this study only pantoyltaurine in a metabolite-antimetabolite ratio of 1:2500 was completely effective as an inhibitor of the growth and acid production of the test organism. The lowest metabolite-antimetabolite ratios, which produced a significant partial reduction of acid formation, were calcium pantothenate: pantoyltaurine 1:500; nicotinic acid: pyridine-3-sulfonic acid 1:1000; and thiamine hydrochloride: pyrithiamine 1:1410. In each instance the degree of inhibition was directly related to the amount of the particular antimetabolite present in the medium.

Conclusions. The findings reveal that pan-

⁸ Landy, M., and Dicken, D. M., *J. Lab. and Clin. Med.*, 1942, **27**, 1086.

⁹ Dreizen, S., and Spies, T. D., *J. D. Res.*, 1947, **26**, 409.

toyltaurine, pyridine-3-sulfonic acid and pyri-thiamine will inhibit the growth and acid production of the *Lactobacillus acidophilus* (Hadley) when present in sufficient concentrations in a medium containing all the known growth essentials of this organism. These metabolic analogues of pantothenic acid, nicotinic acid and thiamine have an affinity for enzymes or for the protein component of enzymes, substrates or the prosthetic groups which they resemble structurally.¹⁰ They tend to replace their corresponding essential nu-

trients in the metabolism of the *Lactobacillus acidophilus* (Hadley) and thereby interfere with its ability to carry on its normal physiological functions. The findings also confirm the previously reported observations that pantothenic acid, thiamine and nicotinic acid are essential for the maximum growth and acid production of the oral *Lactobacillus acidophilus*.

¹⁰ Welch, Arnold D., and Bueding, E., in Green, D. E., *Currents in Biochemical Research*, Interscience Publishers, New York, 1946.

16577

Virus of Eastern Equine Encephalomyelitis Isolated from Chicken Mites (*Dermanyssus gallinae*) and Chicken Lice (*Eomenacanthus stramineus*).*

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Within recent years several neurotropic viruses have been recovered from the chicken mite, *Dermanyssus gallinae* (De Geer), and it has been shown not only to harbor the viruses in nature but to maintain at least one of them by transovarian passage. Smith, Blattner and Heys¹ isolated the virus of St. Louis encephalitis from chicken mites taken near St. Louis during a non-epidemic period, and were able to recover this virus a second time² from mites taken in the same chicken house and from nearby areas. They demonstrated³ the transovarian passage of the St. Louis virus in experimentally infected mites and found⁴ that normal chickens could be infected when bitten by naturally infected *Dermanyssus* and that the virus could then be transmitted to uninfected mites after feed-

ing upon these same chickens. Therefore, the cycle for the St. Louis virus of mite-bird-mite seems to be established. Sulkin⁵ recovered the virus of western equine encephalomyelitis from *Dermanyssus gallinae* obtained on a farm in Dallas County, Texas, where cases of equine encephalomyelitis had occurred. Reeves, Hammon and co-workers⁶ isolated the virus of western equine encephalomyelitis from wild bird mites, *Liponyssus sylviarum*, removed from the nest of a yellow-headed blackbird, *Xanthocephalus xanthocephalus* (Bonaparte), found in Kern County, California. More recently⁷ they have reported on the isolation of another neurotropic virus from mites in this same nest. This strain seems to combine the characteristics of both the western equine and the St. Louis type of encephalitic viruses. Sulkin

¹ Smith, M. G., Blattner, R. J., and Heys, F. M., *Science*, 1944, **100**, 362.

² Smith, M. G., Blattner, R. J., and Heys, F. M., *Proc. Soc. Exp. Biol. and Med.*, 1945, **59**, 136.

³ Smith, M. G., Blattner, R. J., and Heys, F. M., *J. Exp. Med.*, 1946, **84**, 1.

⁴ Smith, M. G., Blattner, R. J., and Heys, F. M., *J. Exp. Med.*, 1947, **86**, 229.

⁵ Sulkin, S. E., *Science*, 1945, **101**, 381.

⁶ Reeves, W. C., Hammon, W. McD., Furman, D. P., McClure, H. E., and Brookman, B., *Science*, 1947, **105**, 411.

⁷ Hammon, W. McD., Reeves, W. C., Cunha, R., Espana, C., and Sather, G., *Science*, 1948, **107**, 77.

and Izumi⁸ recovered the western equine virus from the tropical bird mite, *Liponyssus bursa* (Berlese), taken from a nest containing fledgeling English sparrows, *Passer domestica* (Linn.).

It has been well established, therefore, that bird mites, whether from domestic or wild birds, are capable of harboring in nature at least 3 different strains of encephalitic viruses. It has been determined also that the St. Louis virus may be passed through the egg to the next generation and that it can be transmitted from mite to bird and back again to mite. The present report is to show that yet another strain of virus, that of eastern equine encephalomyelitis, may be found in the chicken mite, *Dermanyssus gallinae*. So far, no one has reported on the recovery of this eastern strain from any arthropod in nature, although several experimental studies have shown that it may be transmitted by mosquitoes in the laboratory. Merrill, Lacaille and TenBroeck^{9,10} were the first to demonstrate transmission of the eastern equine virus by mosquitoes of the genus *Aedes* but not by *Culex pipiens* or *Anopheles quadrimaculatus*. Davis¹¹ likewise demonstrated that the eastern virus could be passed by different species of *Aedes*. No one, however, has worked with the mites.

Isolation of Eastern Equine Encephalomyelitis Virus from Chicken Mites, *Dermanyssus gallinae* (De Geer). During the summer of 1947 sporadic cases of encephalitis in children had been reported from several areas of central Tennessee. Many of the cases were in rural or semi-rural districts and were often associated with the presence of domestic fowl. On August 23, 1947, approximately 300 chicken mites, *Dermanyssus gallinae*, were removed from the roosts of a chicken house near Shelbyville, Tenn. They were placed in

an ampule, frozen in dry ice and returned to the laboratory in Montgomery, Ala., where they were stored in the dry ice refrigerator. On September 16, 1947, the mites were removed from the ampule and triturated in a mortar with 3 ml of buffered saline containing 30% normal rabbit serum. The suspension was then centrifugated for one-half hour at 13,000 rpm in an angle centrifuge placed in the cold room. Twelve-day-old Swiss mice were inoculated with 0.03 ml intracerebrally and 0.1 ml intraperitoneally of the supernatant fluid. Two mice died in three days, one on the fourth and the remaining 5 were sacrificed after 4 days incubation. The brain suspension from one of the dead animals was transferred intracerebrally to other young mice. No bacteria were found in cultures from the brain. Serial passages were made in mice and the animals regularly died or showed convulsions in 2 to 3 days. After 6 transfers in mice the virus gave a titer of $10^{-8.3}$, and was found to pass Mandler and Seitz filters, to be lethal for embryonated eggs in 24 hours and for guinea pigs in 48 hours. The latter showed symptoms of encephalomyelitis.

Neutralization tests against known immune sera were performed for identification of the virus. The technic used was that recommended in the manual of the "Laboratory Methods of the U. S. Army."¹² The new strain was neutralized by the antiserum of the virus of eastern equine encephalomyelitis but not by that of the western equine or of St. Louis encephalitis. Tissue immunity tests were also made by intracerebral inoculation of immune guinea pigs with 0.2 ml of 1,000,000 lethal mouse doses of Lot No. 33 virus. All of the normal control animals and those immune to the western virus died, while guinea pigs immune to the eastern equine strain remained alive. It was thus established that the new active agent was antigenically identical with that of the virus of eastern equine encephalomyelitis. This is the first time that this strain has been recovered from chicken mites taken in nature and also the

⁸ Sulkin, S. E., and Izumi, E. M., *Proc. Soc. Exp. Biol. and Med.*, 1947, **66**, 249.

⁹ Merrill, M. H., Lacaille, G. W., Jr., and TenBroeck, C., *Science*, 1934, **80**, 251.

¹⁰ Merrill, M. H., and TenBroeck, C., *J. Exp. Med.*, 1935, **6**, 687.

¹¹ Davis, W. A., *Am. J. Hyg.*, 1940, No. 2, Sec. C, 45.

¹² Simmons, J. S., and Gentzkow, C. J., *Laboratory Methods of the U. S. Army*, Lea and Febiger, Fifth Ed., 1944.

TABLE I.
Neutralization Tests Against the E.E.E.* (Lot No. 33) Virus.

Location in Tennessee	Date bled	Sera of animals							
		Dog		Cows		Horses		Chickens	
		No.	Pos.	No.	Pos.	No.	Pos.	No.	Pos.
Shelbyville	8-23-47	—	—	4	0	2	0	20	1 pos. 1 wkly. pos.
Alexandria	8-29-47	1	0	3	1	2	0	11	1 pos. 2 wkly. pos.

* Eastern equine encephalomyelitis.

first time that it has been reported in Tennessee. It had been isolated previously from horses in some of the surrounding states: Alabama,¹³ Missouri,¹³ and Michigan.¹⁴

Isolation of Eastern Equine Encephalomyelitis Virus from Chicken Lice. *Eomenacanthus stramineus* (Nitzsch). A few days after the collection of the mites, 23 lice, *Eomanacanthus stramineus* (Nitzsch) (= *Menopon biseriatum* or *M. stramineum*) were removed by means of an aspirator from chickens on a farm near Alexandria, Tenn. These lice were sealed in an ampule, frozen and sent to the laboratory, where they were stored in the dry ice refrigerator. On September 16, 1947, they were removed and treated in the same manner as described for the mites. Eight young mice were inoculated both intracerebrally and intraperitoneally with the supernatant fluid after centrifugation at 13,000 rpm for one-half hour. Five of these died in from 3 to 5 days and 3 were killed on the fifth day. The mouse brains that showed no bacterial growth in broth were passed to other mice, which in turn either died or became ill in 2 days. Six serial passages were made in young mice and then identification of the virus was determined by means of the neutralization test. It also was found to be the virus of eastern equine encephalomyelitis.

Neutralization Tests on Animal Sera. Neutralization tests against the mite strain (Lot No. 33) were performed on sera obtained from animals on the farms at both Shelbyville and Alexandria. The results are

given in Table I. The tests were considered positive if the neutralization index of a serum was 50 or above and weakly positive if it lay between 11 and 49.¹² Antibodies to this virus were found in the sera of 2 out of 20 chickens bled at Shelbyville, although one serum was only weakly positive. Likewise positive tests were obtained on the serum of one cow and 3 out of 11 chickens from the other farm where the lice had been removed from the fowl. Two of the latter sera were only weakly positive.

Discussion. The recovery of the eastern equine strain from chicken lice is not what one would anticipate from their habits. Like all other members of the order Mallophaga, the mouthparts are adapted not for sucking blood but for chewing upon the surface of the skin and feathers. However, Wilson¹⁵ observed a specimen of *E. stramineus* with its mandibles imbedded in a young feather from which the dermal papillae, bearing blood vessels, had not yet withdrawn, and thus drawing and ingesting blood from the wound. Further examination suggested that this type of feeding was habitual. Other means by which lice may become infected with virus are by feeding on blood at the site of traumatic wounds, abrasions, or feeding punctures caused by other arthropods. Therefore, it is possible that these and other species of biting lice may be important vectors of the virus among birds, especially in the absence of other vectors.

The presence of antibodies for the eastern equine strain in a few chicken sera is supportive evidence for finding the virus in the

¹³ Shahan, M. S., and Giltner, L. T., *J. Am. Vet. Assn.*, 1945, **107**, 279.

¹⁴ Brown, G. C., *J. Inf. Dis.*, 1947, **81**, 48.

¹⁵ Wilson, F. H., *Science*, 1933, **77**, 490.

chicken lice, even though they may not be the true vectors. The positive neutralization tests with the cow serum would indicate, however, that some other arthropod, such as the mosquito, is also a vector in this area.

Likewise while the eastern equine virus was recovered from chicken mites in nature, as yet no experimental proof has been given that the virus lives, multiplies and is hereditarily transmitted in these hosts. Further work is being planned in this regard for both species of arthropods.

Summary. The virus of eastern equine encephalomyelitis has been recovered from chicken mites, *Dermanyssus gallinae* (De Geer), and from a mixture of chicken lice, *Menopon pallidum* Nitzsch and *Eomenacanthus stramineus* (Nitzsch), taken in nature

in Tennessee. This is the first isolation of the eastern virus from chicken mites and chicken lice and the first time the virus has been found in this state. Neutralizing antibodies for this virus were present in the sera of a few chickens and one cow. Further confirmation is needed to prove the role of mites and especially of lice as true vectors of this virus.

We wish to express our appreciation to Dr. Amos Christie and Dr. J. C. Peterson of the Department of Pediatrics of the Vanderbilt University School of Medicine whose interest in the encephalitis problem in Tennessee initiated these studies. We wish also to acknowledge the assistance of the Tennessee State Health Department, particularly that of Dr. C. B. Tucker, State Epidemiologist.

16578

Development of Tuberculous Infection. *In vivo* Observations in the Rabbit Ear Chamber.*

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The rabbit ear chamber, first described by Sandison¹ and later modified by Ebert, Florey, and Pullinger,² provides a technic for studying the dynamics of pathological changes in living tissue. In this paper results of studies on tuberculosis by this method are reported.

A thin layer of living vascularized tissue 40 to 50 μ thick can be inoculated with tubercle bacilli directly and examined by frequent daily observations in the unanesthetized animal over a period of weeks. Serial microscopic observations using the highest magnifications are possible, and a graphic record of the findings can be made with

Kodachrome motion pictures. In this way it was possible to record the early development of tuberculous infection. The chamber method is particularly well suited for the study of changes in blood vessels, and striking observations have been made on the vascular reactions in the growing tuberculous lesion.

Materials. The Chamber: Originally observations were made using the modification developed by Ebert, Florey, and Pullinger,² but subsequently a simpler chamber was devised which was smaller and lighter, eliminating the necessity of the cumbersome ear splints and shields used to protect the larger chambers. It consists of a transparent lucite base with a mica coverslip supported by an aluminum ring connected with 3 small bolts (Fig. A).

The base is a highly polished round lucite plate 1 inch in diameter, 1/16 inch thick with a raised central table 1/4 inch in

* This investigation was supported by a research grant from the Division of Research Grants and Fellowships of the National Institute of Health, U. S. Public Health Service.

¹ Sandison, J. C., *Am. J. Anat.*, 1928, **41**, 447.

² Ebert, R. H., Florey, H. W., and Pullinger, B. D., *J. Path. and Bact.*, 1939, **48**, 79.

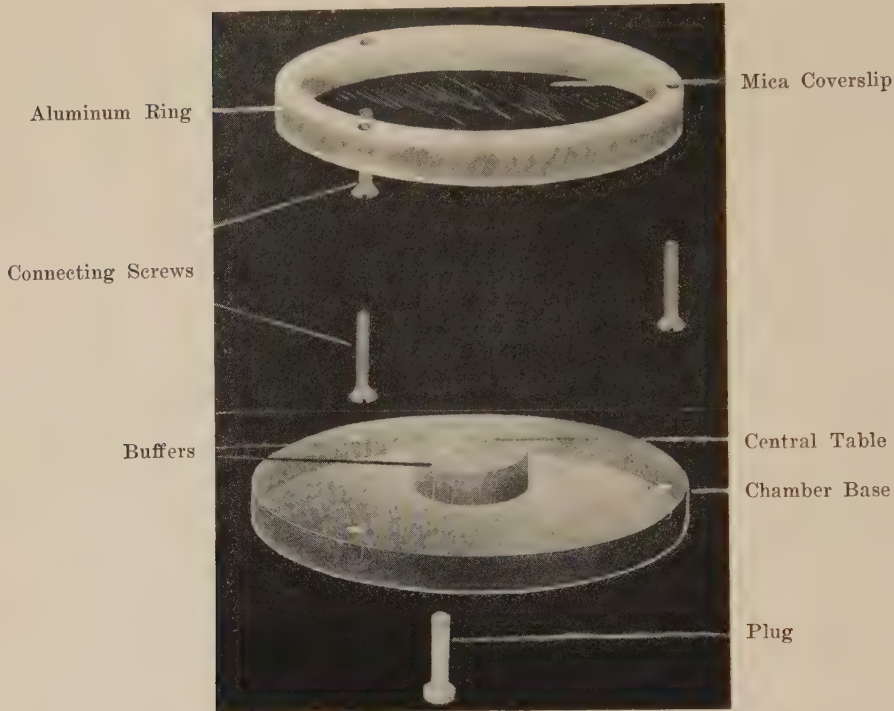


FIG. A.
Exploded diagram of the rabbit ear chamber.

diameter projecting $1/16$ inch above the base plate. A round hole $1/16$ inch in diameter is drilled through the raised central table $1/16$ inch off center to accommodate a fitted lucite plug. Three $1/16$ inch holes are drilled at 120 degree intervals through the base plate, their centers being placed exactly $7/64$ inch from the margin. The holes are countersunk to accommodate the beveled heads of the connecting screws. *The buffers*² are glued to the margin of the table surface on a radius passing through the connecting screws. They are cut from a thin sheet of lucite, and should be approximately $40\ \mu$ thick. *The plug* is machined from lucite and has highly polished ends. It has a flange on one end approximately $1/32$ inch thick and $1/8$ inch in diameter. It is approximately $1/16$ inch in diameter and is tapered (very slightly) away from the flanged end so that when fully inserted it fits tightly. It is approximately $5/32$ inch in overall length, being long enough to protrude $2/1000$ inches above the surface of the central table when inserted.

The coverslip is made of selected clear mica split to a thickness of approximately $40\ \mu$. It is supported by an aluminum ring $7/8$ inch outside diameter and $11/16$ inch inside diameter, thus being $3/32$ inch wide. It is $1/16$ inch thick and has 3 threaded holes placed centrally at 120 degree intervals to receive the connecting screws. These measurements allow the oil immersion objective to be brought into focus over the entire central table without striking the aluminum ring and yet afford sufficient strength with minimum weight. The mica is cut into a $7/8$ inch circular sheet and glued to the aluminum ring with any firm adherent (we have found Permunt satisfactory), and 3 holes are punched over the threaded holes in the aluminum ring. *The connecting screws* are brass 0-80 machine screws $3/16$ inch long used to attach the base to the coverslip.

A specially designed movable stage similar to those previously described² is used to hold the ear chamber during observations.

Methods. Insertion of the Chamber: Un-

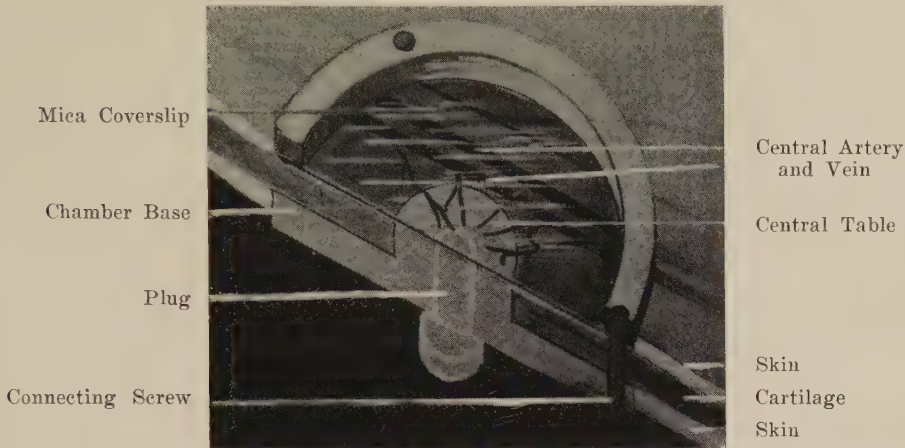


FIG. B.
Cross section diagram of a chamber in a rabbit's ear.

der aseptic conditions 4 holes are punched near the end of the pinna of a rabbit's ear—one central hole and 3 peripheral smaller holes. The skin is removed from both sides of the cartilage over a circular area including all of the holes. The chamber base is then placed with its raised central table protruding through the central hole, the 3 connecting bolts passing through the peripheral holes, and the coverslip is screwed in place on the opposite side of the cartilage. By careful adjustment of the connecting bolts the coverslip is brought into contact with the 3 buffers on the surface of the central table. This leaves a sterile film of blood 40 to 50 μ thick overlying the central table visible through the mica coverslip into which vessels may grow from the vascular bed of the adjacent cartilage. Within 14 to 28 days the area becomes completely vascularized (Fig. B), and the vessels subsequently become differentiated into arterioles, venules, and capillaries.³

Inoculum. Bovine tubercle bacilli (Strain Ravenel RV) grown in Dubos liquid medium for one week were used in this experiment. After the culture was centrifuged and the supernatant liquid decanted, the bacilli were

resuspended in normal saline; the weight of bacilli was calculated from turbidimetric readings of the original culture.

Technic of Inoculation. A red blood cell pipette (previously calibrated) with a heat-drawn tip was used to introduce the suspension of tubercle bacilli. The plug was removed aseptically,² and the fine tip of the pipette placed well into the plug hole; then 0.001 to 0.003 ml was slowly introduced. Although the method is crude, it allows for a rough approximation of the given figures and was found adequate for the purpose of this experiment.

Results. Four chambers were studied in detail from the time of tissue inoculation till the development of necrosis. A motion picture was taken of only one complete experiment over a period of 30 days, but in all experiments (with one exception discussed below) the results were essentially the same.

The Normal Chamber. The growth of new tissue onto the central table and the development of what Clark has called the "adult pattern" has been described in detail elsewhere.³ The adult pattern refers to certain characteristics of appearance and behavior of mature connective tissue on the observation area after it has become stabilized. In summary, these characteristics are as follows: The vessels are well differentiated into arter-

³ Clark, E. R., Hitschler, W. J., Kirby-Smith, H., Rex, R. O., and Smith, J. W., *Anat. Rec.*, 1931, 50, 129.

ioles, venules, and capillaries. The muscular walls of the arterioles are clearly visible and are seen to contract rhythmically in the normal chamber. Contraction of the main arterioles supplying the tissue over the observation area causes temporary emptying of most of the vessels on the table. All vessels have a definite tonus, and the vascular endothelium is sharply defined. Usually only a portion of the vessels on the central table are filled with blood at any one time, and "plasma skimming"⁴ is a common phenomenon. In the normal vessel the flow of red blood cells is streamlined if rapid enough, and white blood cells tend to be thrown toward the periphery of the stream. Often white blood cells roll along the endothelium at a much slower rate than that of the flow at the center of the vessel. If a physical or chemical agent causes injury to the vascular endothelium, it becomes "sticky", and instead of rolling along the endothelial wall leukocytes adhere to it. Sticking of leukocytes to endothelium is one of the earliest signs of inflammation in living tissue and precedes diapedesis of cells. A fairly heavy connective tissue stroma supports the vessels and no free fluid is seen. Extravascular cells are numerous in the growing chamber but are considerably reduced in numbers in the normal adult chamber. Histiocytes are seen scattered throughout the connective tissue and are particularly numerous along vessels. Adventitial cells which do not take up vital stains are seen along vessels. Fibroblasts and fibrocytes are not visible normally but stain faintly with supravital stains. Rare polymorphonuclear leukocytes and mononuclear cells are seen. Lymphatics are present in some chambers, but the lymph flow is very sluggish, and often only pulsation of fluid and cells can be seen. The "normal chamber" is an ideal which cannot be attained since accidental mechanical trauma may cause mild temporary inflammatory changes. On rare occasions small hemorrhages are seen, and there may be a temporary localized sticking

of leukocytes to vascular endothelium. For the purpose of these experiments, however, chambers were chosen which showed a stabilized adult pattern and were as normal as seems to be possible.

Mechanical Trauma Due to Inoculation.

The inflammatory changes associated with withdrawal of the plug, inoculating fluid, and reinserting the plug have been studied in detail in the past,² and although the extent of the trauma may vary considerably, the duration of change can be predicted with a fair degree of accuracy.

There is always some localized sticking of leukocytes in vessels near the plug margin, and localized collections of polymorphonuclear leukocytes and occasional mononuclears are visible for several days after pulling the plug. Localized stasis, thrombosis, and hemorrhage may occur, but vascular changes are temporary and there is a return to normal after 3 to 4 days. Small hemorrhages disappear in 3 to 5 days. In the 4 chambers studied in these experiments the traumatic changes varied from well localized cellular sticking to stasis and thrombosis of a few vessels and localized hemorrhage around the plug margin.

The size of the inoculum varied from 0.000001 mg to 0.0015 mg. The larger inoculum was used in "injection chambers"⁵ in which injections were made via a duct built into the lucite base. In this type of chamber part of the inoculum is unavoidably lost in the large injection duct.

Early Reaction. For descriptive purposes the response of the tissue in the observation area to the presence of tubercle bacilli has been divided into an early and a late reaction. The early response was minimal and lasted 9 to 23 days. During the first few days it was indistinguishable from the inflammatory change associated with pulling the plug.

In all cases the vascular reaction was exceedingly mild. (Fig. C2-4) Very slight dilation of vessels and localized cellular sticking was the essential reaction which developed during the initial period of 6 to 7 days;

⁴ Krogh, A., *The Anatomy and Physiology of the Capillaries*, 1929, Yale University Press, New Haven.

⁵ Ebert, R. H., Sanders, A. G., and Florey, H. W., *Brit. J. Exp. Path.*, 1940, **21**, 212.

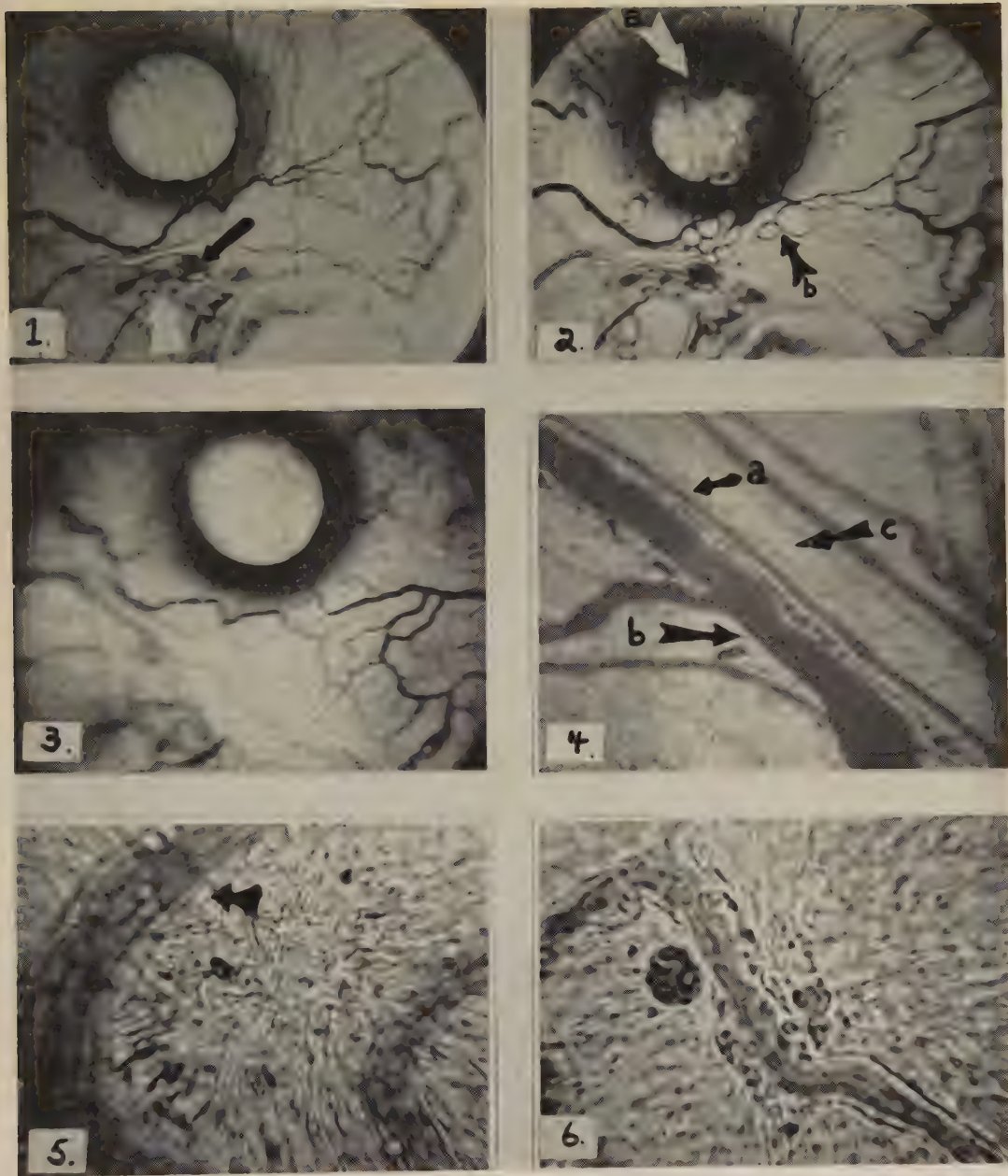


FIG. C.

The development of tuberculous infection illustrated by frames taken from a kodachrome motion picture of a single experiment.

1. 18X. One day prior to inoculation. The tissue is essentially normal except for a small hemorrhage produced by accidental trauma (see arrows).

2. 18X. Ten minutes after the inoculation of 0.0004 mg of virulent bovine tubercle bacilli (Strain Ravenel RV). There is hemorrhage around the plug (a) caused by the procedure. Note the air bubbles below the plug (b) introduced when the plug was reinserted. There is little vascular damage.

3. 18X. Third day after the inoculation. The hemorrhage around the plug has cleared and the air bubbles have disappeared. The vessels are normal.

4. 100X. Third day. An arteriole (a), venule (b), and lymphatic (c) appear normal.

5. 400X. Tenth day. This is the first vascular reaction seen. There is sticking of white cells to vascular endothelium (arrow). A tuberculin test (1:10 O.T.) set on the tenth day was read as weakly positive on the twelfth day (a 1:10 O.T. test on the sixth day was negative).

6. 400X. Eleventh day. There is marked sticking of white cells to vascular endothelium.

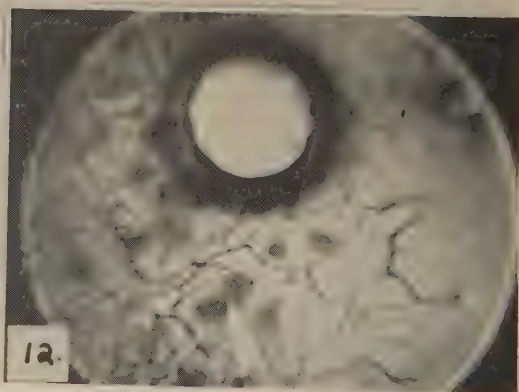
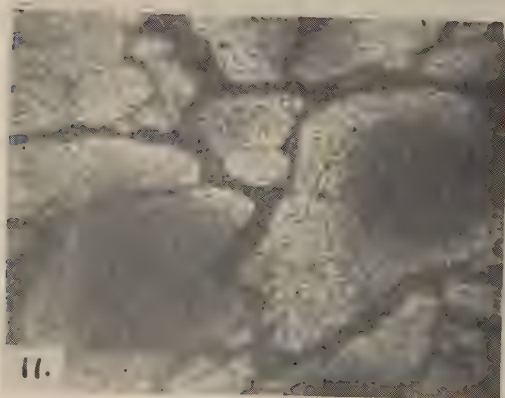
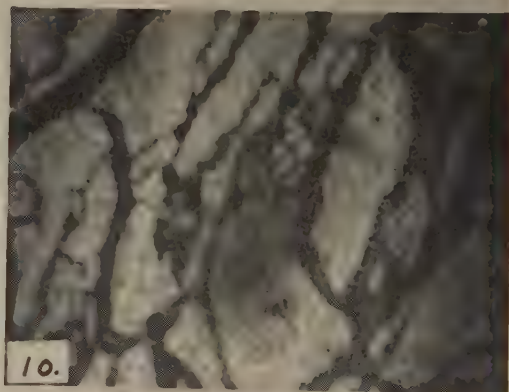
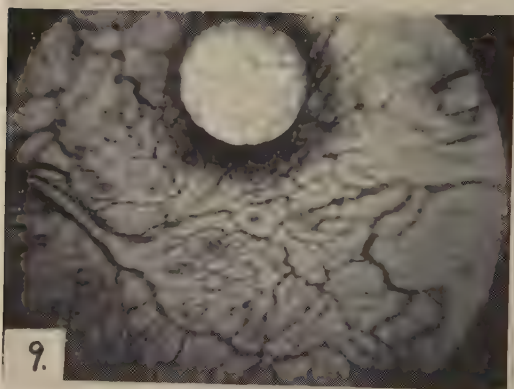
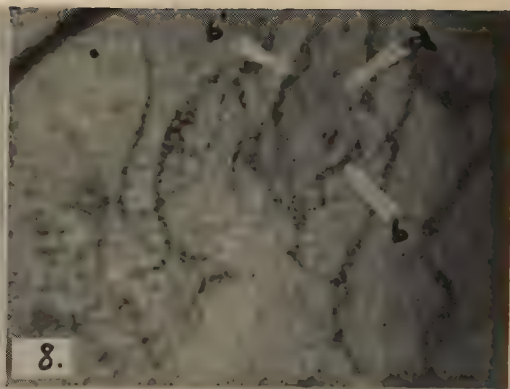
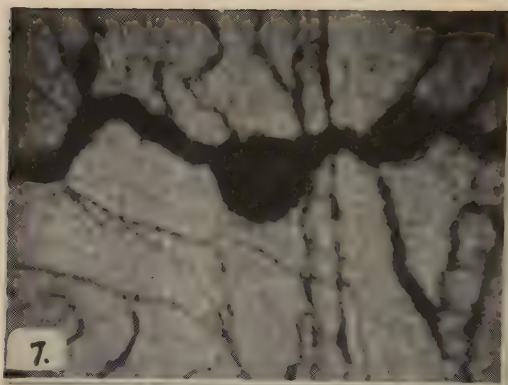


FIG. C (continued).

7. 100X. Eleventh day. There is aneurysm formation in a venule due to vascular endothelial damage.

8. 100X. Fifteenth day. A small area of infiltrate can be seen (a) and adjacent to it are 2 small thrombosed vessels (b and b'). These were the first thromboses seen.

9. 18X. Seventeenth day. There is marked dilatation of all vessels and several areas of increased density can be seen centrally located. These are regions of dense cellular infiltration.

10. 100X. Eighteenth day. A small area of cellular infiltration surrounded by multiple dilated vessels.

11. 100X. Twentieth day. Two areas of infiltrate surrounded by dilated markedly damaged vessels. The blood flow in these vessels was sluggish and pulsating and there was hemo-concentration.

12. 18X. Twenty-first day. There has been an increase in the size and number of tuberculous infiltrates. Most of the vessels on the right side of the table and over the plug are thrombosed.

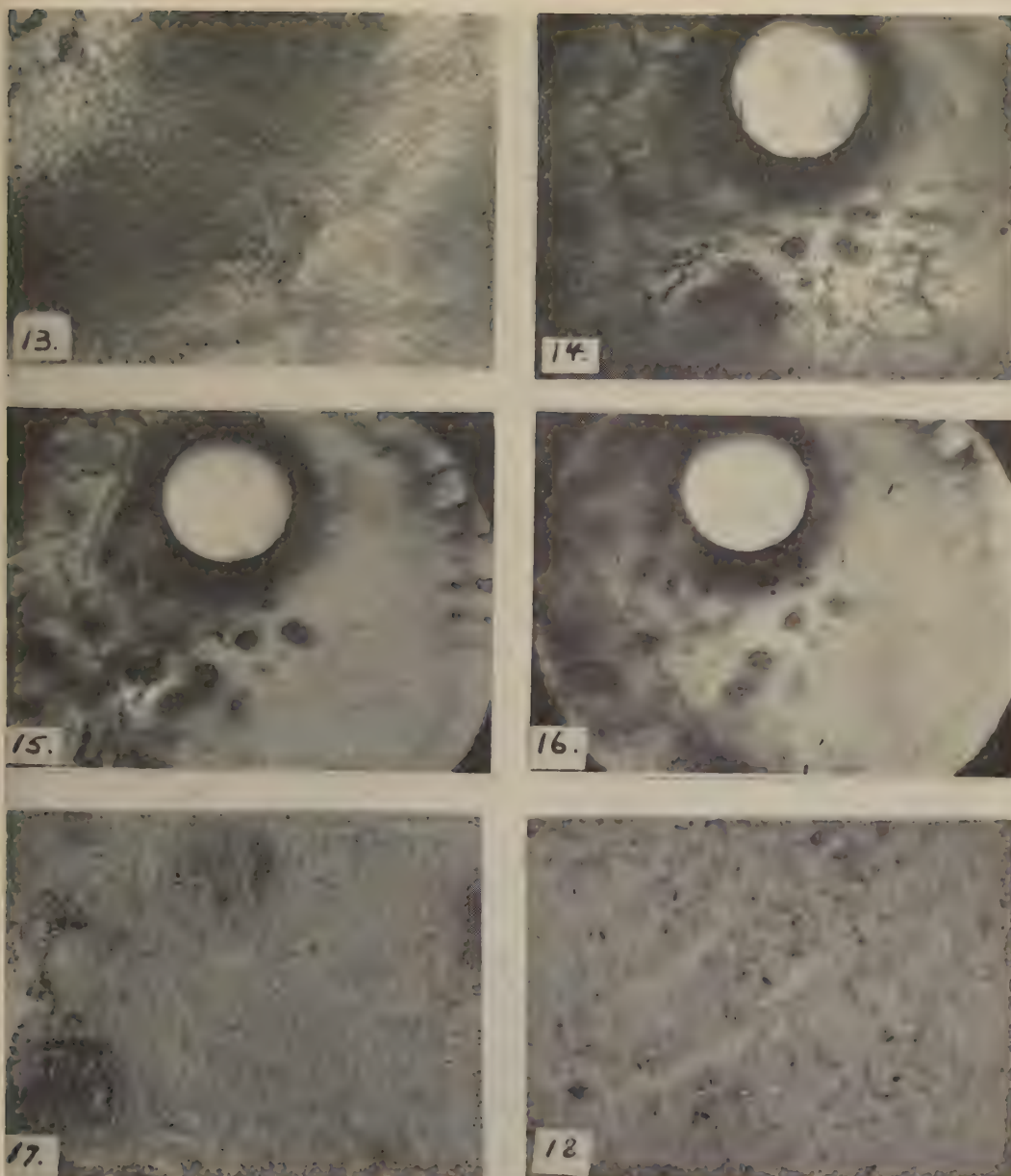


FIG. C (continued).

13. 100X. Twenty-first day. This is the same area photographed in Fig. C-11 and pointed to by the arrow in Fig. C-12. The dense regions have become confluent and there has been complete thrombosis of the vessels surrounding the infiltrates.

14. 18X. Twenty-third day. There is increased confluence of the dense areas of tuberculous infiltrates and extension of thrombosis.

15. 18X. Twenty-fifth day. The thrombosed vessels on the right side of the table seen in Fig. C-14 have disappeared and the area is necrotic. Only a small vascular area remains along the left border of the observation table. The remaining vessels are dilated and show evidence of damage.

16. 18X. Twenty-ninth day. There has been almost complete destruction of all the vessels on the table. The animal was sacrificed immediately after this photograph was taken and the tissue fixed by injection of the central artery with alcohol.

17. 100X. A hematoxylin and eosin section showing tuberculous caseation. The dense areas are the same as those seen *in vivo*.

18. 950X. Numerous acid-fast bacilli are seen. This is a section through the region of density seen in Fig. C-13. All areas of tuberculous infiltration were loaded with tubercle bacilli. Only rare bacilli were found between these areas.

these changes were so mild that in the early experiments it was doubted that infection had actually occurred. In one case reinoculation was carried out on the 5th day because the tissue apparently had returned to normal. In two cases localized free fluid persisted after the inoculation with tubercle bacilli. In one case this fluid persisted till the development of the "late reaction", and in the other it disappeared 8 days before the "late reaction" developed.

In 2 experiments there was very little cellular exudate—not much more than could be accounted for by the mechanical trauma. In the 2 cases in which fluid was seen mononuclear cells and macrophages were found in the fluid, and in these experiments as well as one other localized collections of macrophages were observed after the 9th day.

Late Reaction. In contrast to the relatively mild early reaction there occurred sometime between the 10th and 24th day a much more explosive sort of tissue response. The change was sudden and progressive. It began with marked sticking of white cells to the vascular endothelium (Fig. C5, 6) accompanied by considerable infiltration of white cells into the tissue. This was followed by progressive dilation of vessels until they seemed to have lost all tonus (Fig. C-9,10). Associated with this dilation the vessels became tortuous, and in one case small aneurysmal dilations occurred in the wall of 2 venules (Fig. C-7). The vascular endothelium no longer appeared as sharply defined as it did in the normal vessels. As was mentioned above, only a portion of the vessels in the normal chamber appeared filled with blood at any one time but now all vessels were filled and blood flow became sluggish and pulsating. Agglutination of red blood cells was noted, but sludging was not as prominent as has been described by Knisely and associates in other disorders.^{6,7} Simultaneously with these vascular changes there occurred a

considerable increase of the amount of exudate (Fig. C-8), and free fluid was seen in all chambers. For the most part the exudate was composed of monocytes, lymphocytes, and macrophages but some polymorphonuclear leukocytes were seen. Occasionally giant cells could be distinguished. There was considerable increase in the size of the localized collections of cells mentioned in the early reaction, and these areas became so packed that individual cells were seen with difficulty. Stasis and localized thrombosis of small venules surrounding these dense collections of cells then developed (Fig. C8-11), and this phase was rapidly followed by extensive thrombosis of surrounding vessels (Fig. C12-16).

The sequence of events in a thrombosing vessel could be followed in the greatest detail. First, there appeared to be hemoconcentration in the dilated venule; then red cells became so densely packed that the margins of individual cells were no longer visible. Some pulsation continued for a time in the degenerating vessel, and it was common to see a thrombosing vessel branching from another venule in which flow was still present. Gradually the endothelium became less distinct and finally the vessel disappeared. The rate at which these changes occurred varied. In some chambers it occurred rapidly in about 6 to 7 days; in others, more gradually over a period of 19 days—but in every case except one the result was the same: progressive thrombosis of blood vessels until almost all the visible tissue was avascular and necrotic (Fig. C-16). In the one exception the explosive reaction began on the 15th day and gradually progressed till the 22nd day after inoculation. At this time there was very marked dilation, considerable exudate, and several areas of venous thrombosis. Between the 22nd and 27th days the process remained relatively stationary, and from the 28th till the 35th day there was a gradual return to normal with considerable reabsorption of the exudate. A tuberculin test on the 46th day with 1:10 OT was negative.

Tuberculin tests at intervals were carried out in only one rabbit. In this animal the

⁶ Knisely, M. H., Eliot, T. S., and Bloch, E. H., *Arch. Surg.*, 1945, **51**, 220.

⁷ Knisely, M. H., Stratman-Thomas, W. K., Eliot, T. S., and Bloch, E. H., *J. Nat. Malaria Soc.*, 1945, **4**, 285.

first tuberculin test (1:10 OT) on the 6th day was negative. The second, on the 10th day was weakly positive; a third on the 27th day was strongly positive. The marked tissue response began on the 10th day simultaneously with the development of skin hypersensitivity and progressed to almost complete dissolution of the visible tissues on the 29th day (Fig. C-16).

Stained Sections. At the end of the experiments the observation area and surrounding tissue was fixed in alcohol or ice-cold acetone, and serial sections were made with both hematoxylin and eosin and acid-fast stains. In 3 cases almost the entire area of tissue over the central table was necrotic (Fig. C-17) and numerous acid-fast bacilli were found in the areas of dense infiltrate noted in the living animal (Fig. C-18). Scattered acid-fast bacilli were found elsewhere, but nowhere were they as numerous as in the areas of heavy infiltration. Tubercles consisting of areas of central necrosis, and containing acid-fast bacilli, surrounded by epithelioid cells and giant cells were found in the connective tissue adjacent to the central table. In the fourth case the tissue was removed from the animal in which the chamber had shown a return toward normal. In these sections epithelioid cells and giant cells were found but no acid-fast bacilli could be discovered after careful search.

Discussion. The most striking observation made in the course of these experiments was the vascular reaction to the presence of tubercle bacilli in the connective tissue. Although tuberculin testing was done systematically in only one experiment, the exact correlation of developing skin hypersensitivity and vascular changes in this one case seems more than chance occurrence. During the early stages the reaction was little more than what one would expect from a benign foreign body, but the development of hypersensitivity ushered in a series of destructive changes in the vascular tree of the observation area. It is impossible to say if these

changes in vascular endothelium were primary or secondary, but regardless of their exact origin they certainly play an important part in the development of tissue destruction. In effect we observed a series of small infarcts. It should be remembered that the thrombosis described as occurring was in relatively small vessels—none larger than 30 to 40 μ in diameter normally (although some were considerably more dilated at the time of thrombosis). In other words, this was microscopic thrombosis and did not involve any vessel of appreciable size.

One hesitates to infer that similar changes occur in all tissue and in all animals as a part of the pathogenesis of the caseous tubercle. It was a striking fact, however, that in the stained sections one found no indication of the dramatic vascular changes which had occurred in the living animal and could see nothing but necrotic tissue surrounded by tuberculous tissue.

Summary. A new use of the rabbit ear chamber technic is described.

Microscopic studies of the tissue changes in developing tuberculous infection within the chamber were made. The early reaction to the presence of living tubercle bacilli was minimal. The late reaction was an explosive, necrotizing response. In the one rabbit systematically skin tested with Old Tuberculin this late reaction began coincidentally with the development of skin hypersensitivity. Progressive vascular damage ending in venous thrombosis played an important role in tissue destruction.

The experimental technic and microscopic observations described have been recorded in Kodachrome motion pictures. It is suggested that this technic may be used for microscopic study of the dynamics of tissue changes in other pathological conditions.

Grateful acknowledgment is made to Drs. M. H. Knisely, E. H. Bloch, and Miss Louise Warner for their generous loan of equipment and invaluable technical advice.

Dermal Hypersensitivity to Toxoplasma Antigens (Toxoplasmins).

J. K. FRENKEL.* (Introduced by James F. Rinehart.)

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Both the toxoplasma-neutralizing antibody test¹ and the complement fixation test² have been used in patients to demonstrate specific antibodies to toxoplasma, indicative of past and possibly persistent infection. As pointed out by Sabin and Ruchman,¹ the neutralizing antibodies are unstable both at room and refrigerator temperatures, necessitating storage of test sera in the frozen state. Diagnostic readings require a week to develop. Equivocal results are frequently obtained, presumably due to low titers of neutralizing antibodies.³ For performance of the test an active strain of toxoplasma must be maintained in the laboratory. While the toxoplasma-neutralizing antibodies seem to persist for a matter of years in the experimentally infected animal, complement-fixing antibodies appear to be more evanescent.² Many human cases giving a positive neutralizing-antibody test have been encountered where the complement fixation test has been negative. The presence of complement-fixing antibodies may be more indicative of a recent infection or of recent contact with toxoplasma antigen.

It appeared desirable, therefore, to investigate the possibility of developing toxoplasma antigens for skin tests which would be simple, rapid and less costly in performance. The preparation of several antigens will be described and preliminary data on their use in patients are given.

Experimental: (a) *Chick-embryo antigens:* It was felt originally that toxoplasma grown in the developing chick might give useful antigens, at the same time almost excluding the possibility of accidental infection

of the host with other microorganisms. Sabin's RH strain⁴ of toxoplasma was inoculated onto the freshly collapsed chorioallantoic membrane of the 7 to 9 day old chick embryo. These membranes were harvested 4 or 5 days after inoculation, which was about a day before the expected death of the embryo. The membranes were ground with alundum. The pulp was diluted with normal saline to one-tenth its concentration, then frozen in a carbon dioxide ice-alcohol mixture and thawed, repeatedly. The resulting material was centrifuged and the sediment discarded. The supernatant fluid was preserved with phenol 1:1000 or merthiolate 1:10,000 and then passed through a Seitz filter. Uninfected membranes were treated similarly and the product was used as control antigen.

Such filtered supernatant fluids were tested for potency in about 70 guinea pigs which had a chronic latent infection with toxoplasma. It was found that 1/10 cc of a 1 to 50 dilution of the test antigen would produce areas of erythema and induration of about 30 and 15 mm, respectively, after 24 hours, in infected animals, but erythema of not more than a few millimeters in normal control guinea pigs. Permitting the uncentrifuged membrane suspension to autolyse in the refrigerator for a few days slightly increased the titre of the test antigen. The control antigen gave rise to less than 5 mm of erythema in both normal and immune guinea pigs.

Chick-embryo toxoplasma and control antigens were employed in skin testing of patients during 1945-1947. Again 15+ mm of induration at the site of the test dose with not more than 4 mm of induration at the site of the control injection was considered a positive test. Most of the positive tests were apparent after 24 hours, with occasional reactions tak-

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¹ Sabin, A. B., and Ruchman, I., *Proc. Soc. Exp. Biol. and Med.*, 1942, **51**, 1.

² Warren, J., and Sabin, A. B., *Proc. Soc. Exp. Biol. and Med.*, 1942, **51**, 11.

³ Heidelman, J. M., *Arch. Ophth.*, 1945, **34**, 28.

⁴ Sabin, A. B., *J. A. M. A.*, 1941, **116**, 801.

ing 48 hours to develop. The latter time was therefore used routinely to read the tests. A positive test persisted usually for 4 to 10 days, while the erythema of the negative and the control tests, in most cases, had disappeared in 24 hours.

(b) *Hamster and Mouse Antigens:* Antigens from the sediment of peritoneal exudate of heavily infected hamsters and mice were developed in 1947 and largely replaced the chick-embryo antigen. In infected chick embryos toxoplasma were far in the minority, compared to the number of host cells as shown on sections through the chorioallantoic membranes. Consequently antigens prepared from chorioallantoic membranes contained a disproportionately large amount of chick embryo protein. Exudates of mice and hamsters 4 or 5 days after intraperitoneal infection with the RH strain of toxoplasma, contained leukocytes and organisms in about equal proportions. Toxoplasma were estimated to make up approximately one quarter of the volume of peritoneal exudate sediments. The latter gave positive skin reactions in infected animals and in patients in dilutions of 1:500 to 1:10,000. Hence, these antigens were of greater potency and they were associated with less carrier-protein.

Toxoplasma antigens derived from mouse peritoneal exudates are preferable to those from hamsters since the average yield of fluid and of organisms is greater from adult mice than from adult hamsters. The mice to be used for antigen preparation must be known to be free from paratyphoid and other pathogens.

Preparation of Toxoplasmin from Peritoneal Exudates. Mice (and similarly, hamsters) are injected intraperitoneally with 0.2 cc of a 1:50 dilution of peritoneal fluid from a mouse which had been infected intraperitoneally with the RH strain of toxoplasma. Four or 5 days after injection their abdomen becomes distended with peritoneal fluid. Anticipating their expected death from toxoplasmosis, mice are lightly etherized and the peritoneal fluid is aspirated through a previously disinfected area with a No. 20 needle fitted to a 5 cc syringe. The fluids obtained, which

are rich in fibrinogen, are pooled in previously weighed centrifuge tubes containing enough heparin to make a final concentration of about 10 mg%. After thorough mixing the pooled fluids are centrifuged for 20 minutes at about 2000 RPM. The supernatant fluid is discarded since it is poor in antigenic material relative to its protein content. Then the tube is reweighed to determine the amount of sediment remaining. The latter corresponds to approximately 1% of the original fluid weight. The sediment is resuspended in 10 times its weight of saline. The suspension is then exposed in a watch glass to a General Electric germicidal lamp of 15W at a distance of 30 cm for 4 minutes or to an equivalent amount of ultra violet germicidal energy. The depth of the suspension should not exceed 2-3 mm. Both toxoplasma and accidental contaminants will be killed by this treatment. The 1:10 suspension is frozen rapidly in a carbon dioxide ice-alcohol mixture and thawed alternately for 5-10 times to break up the organisms. Subsequently a 1:1000 dilution is prepared with normal saline containing 0.25% phenol as preservative. Its protein content is in the neighborhood of 2.2 mg%. An injection of 0.1 cc of a 1:1000 dilution of mouse toxoplasmin contains approximately 8,000 autolysed organisms.

For the preparation of the control antigen mouse spleen appeared preferable to sterile peritoneal exudates produced by irritants. The latter were difficult to produce in amounts necessary to recover sufficient cell sediment. The complete disappearance time of the irritant injected was hard to determine with accuracy. In contrast to this, ground spleen suspensions are easily prepared. Their cell constituents are not vastly different from those of the peritoneal exudate after infection, which contains about 40% of macrophages, 30% of monocytes and lymphocytes and 30% of heterophile granulocytes, and variable amounts of red blood cells apart from toxoplasma. Hence the control antigen is prepared from ground normal mouse spleen and the pulp is treated like peritoneal fluid sediment.

(c) *Assays of the Antigens.* The antigens

are subjected to tests for non-toxicity and sterility including aerobic and anaerobic cultures, and the intracerebral inoculation of hamsters and mice. These animals should succumb to a challenge injection performed 3 weeks later. Seitz-filtration was not used since it removes much antigenic material.

Preliminary potency tests are performed on guinea pigs having chronic latent toxoplasmosis. Later the material is checked on known toxoplasmin reactors, also known to have neutralizing antibodies to toxoplasma and on non-immune human beings. Criteria for reading of the tests are similar for chick embryo, mouse or hamster antigens. It should be remarked here, that guinea pigs may quickly acquire dermal hypersensitivity to the carrier-protein, so that they should not be used for more than 2 or 3 skin tests. Human beings have not been observed to acquire dermal hypersensitivity to chick embryo, mouse, hamster or toxoplasma proteins, in result of repeated skin testing. Some infected guinea pigs do not seem to be hypersensitive to toxoplasmin. Most patients show more violent reactions to toxoplasmin than guinea pigs. For these reasons clinical assay of the antigens is necessary. A 1:1000 or 1:10,000 dilution of peritoneal exudate sediment was found to be a satisfactory antigen for use in patients. A test was considered positive if 10-30 mm of induration and 10-50 mm of erythema were present after 48 hours. The control injection rarely resulted in more than 3 mm of erythema at the end of 48 hours. Certain laboratory workers exhibited larger dermal reactions to injections of the control antigen, which appeared more rapidly and disappeared more quickly than a positive toxoplasmin test. Positive controls were more frequently encountered after the use of chick embryo than after employment of mouse antigens. Furthermore, mouse antigens gave rise to more marked quantitative differences between test and control antigen injections that were positive.

Antigenic potency was retained for periods of from 5 to 16 months, at which time all the material had been used. Antigen suspensions increased in potency upon storage, sug-

gesting further disintegration of the previously broken-up toxoplasma into a more soluble protein.

(d) *Correlation of Dermal Hypersensitivity with the Presence of Neutralizing Antibodies in Patients.* Neutralizing antibodies are demonstrated by their inhibiting effects in toxoplasma-serum mixtures upon the development of papulonecrotic skin lesions in rabbits, at the sites of intradermal injection of various dilutions of toxoplasma. Neutralizing antibody tests were conducted essentially as described by Sabin and Ruchman.¹ The following modifications were used: 1. Peritoneal exudate of an intraperitoneally infected mouse was used instead of infected brain suspension. 2. The peritoneal exudate, containing many toxoplasma organisms, was diluted in normal saline rather than in Tyrode's solution. 3. Eight instead of 4 tests were performed on one rabbit. 4. Instead of tracing the lesions for the final reading, the rabbit skin was spread out on a glass plate and read by transillumination. This type of reading not only takes into account the linear extensions of the inflammatory process but also evaluates the intensity of the inflammatory lesion in depth. Each test skin bearing 8 separate tests was photographed by transmitted light and then fixed in formalin.

Patients who were skin tested repeatedly with negative results did not develop toxoplasma neutralizing antibodies. However, patients in whom a positive skin reaction was elicited, frequently did show an anamnestic rise in neutralizing antibody titre.

(e) *Correlation of Dermal Hypersensitivity with the Presence of Complement-Fixing Antibodies in Patients.* Duplicate specimens of sera investigated for neutralizing antibodies were tested for the presence of complement-fixing antibodies. These tests were conducted by Dr. Carl M. Eklund and Dr. David Lackman of the Rocky Mountain Laboratory, Hamilton, Montana, and will be reported at a later date. Two of their findings, however, should be quoted here. In the first place, patients who were skin tested repeatedly with negative results being obtained did not develop complement-fixing

TABLE I.

Comparison of Results Obtained by the Use of Toxoplasmin Skin Tests and Toxoplasma Neutralizing Antibody Tests in 108 Patients.

Neutralizing antibody test		Skin test		
		Positive	Negative	Equivocal
Positive	47	44	3	0
Negative	43	3	40	0
Equivocal	18	12	6	0
Total	108	59	49	0

TABLE II.

Percentage Correlation Between Toxoplasma Neutralizing Antibody Tests and Toxoplasmin Skin Tests Based on Data Incorporated in Table I.

Neutralizing antibody test		Skin test		
		Positive	Negative	Equivocal
Positive	%	%	%	
Negative	100	94	6	
Equivocal	100	7	93	
	100	67	33	

TABLE III.

Percentage Correlation Between Toxoplasmin Sensitivity and Results of Toxoplasma Neutralizing Antibody Tests Based on Data Presented in Table I.

Neutralizing antibody test	Skin test		
	Positive	Negative	Equivocal
Positive	%	%	
Negative	75	6	
Equivocal	5	82	
	20	12	
Total	100	100	

antibodies to toxoplasma antigen. Secondly, a positive toxoplasmin skin test did give rise to complement-fixing antibodies in a great majority of my patients so investigated.

Results. (a) *Comparison of data obtained by use of toxoplasmin skin tests and neutralizing antibody tests.* Table I presents the results obtained by the 2 tests in 108 patients. Table II indicates that a high percentage of analogous results are obtained with skin test, if the results of the neutralizing antibody tests are clearly positive or negative. Two thirds of the equivocally neutralizing sera were accompanied by positive skin tests and one third by negative ones. Table III illustrates in toxoplasmin positive and toxoplasmin negative patients, the scattering of

results obtained with the neutralizing antibody test.

The sera of 3 toxoplasmin positive patients did not show toxoplasma neutralizing antibodies at the time of the initial skin test. However, neutralizing antibodies were found upon retesting with the identical serum specimen in one and with the second specimen in 2 of the patients. Furthermore, positive skin tests were obtained with skin testing material derived from chick embryo and hamster or mouse in each of the 3 patients. Hence these 3 negative neutralizing antibody tests must be considered to have been "falsely negative" ones. Two of the patients presented themselves with lesions of chorioretinitis, while a third one did not exhibit clinical signs suggestive of toxoplasmosis.

The 3 toxoplasmin negative patients whose sera contained toxoplasma neutralizing antibodies were not available for restudy. It is possible that their skin tests became positive after the 2 day observation period. A late appearance of signs of dermal hypersensitivity was observed in an occasional patient. None of the 3 patients showed clinical signs suggestive of toxoplasmosis.

The occurrence of an appreciable percentage of equivocal results is an objectionable feature of the neutralizing antibody test. Since a skin test acts as a booster injection, the rise in neutralizing and complement-fixing antibody titres following it, served as convenient evidence in favor of the specificity of an associated positive skin test. No such anamnestic rise has been noted, associated with a negative skin test. Hence most equivocally neutralizing antibodies appear to be due to low-titre neutralizing antibodies. Six of the 12 toxoplasmin positive patients either showed clinical signs suggestive of toxoplasmosis or were parents of toxoplasmic infants. None of the 6 toxoplasmin negative patients belonged in either of these groups.

(b) *Toxoplasmin sensitivity in certain selected and unselected individuals (Table IV).* The incidence of toxoplasmin sensitivity in a group of younger adults (medical students, laboratory and office workers), aged 20-35 years, was 10%. In an older group of

TABLE IV.
Toxoplasmin Sensitivity in Certain Selected and Unselected Individuals.

Grouping	Years of age	No.	Toxoplasmin-		
			Positive	Negative	Equivocal
			%	%	%
1. Survey—unselected		100	19* 19	81 81	
1a. " "	20-35	50	5 10	45 90	
1b. " "	50-83	50	14 28	36 72	
2. Patients with chorioretinitis†	Mean 23	28	20 71	8 29	
2a. Mothers of toxoplasmin positive patients		8	8 100		
2b. Mothers of toxoplasmin negative patients		2	1 50	1 50	
3. Patients with anterior uveitis†	Mean 35	40	13 33	27 67	
4. Infants with congenital toxoplasmosis, fatal, toxoplasma isolated	5 weeks	2		1 50	1 50
5. Infants with neonatal toxoplasmosis, surviving	Mean 2	2	2 100		
6. Mothers of No. 4, 5, and others	" 30	6	6 100		
7. Fathers of No. 4, 5, and others	" 30	4	2 50	2 50	
8. Siblings of No. 4, 5, and others	" 6	7		7 100	
9. Pathologists	" 36	7	4 57	3 43	
10. Patients with hydrocephalus without chorioretinitis	" 1	10	1 10	9 90	

* The first numeral refers to the number of toxoplasmin positive patients, the second to the percentage within the total group.

† Patients seen in conjunction with a study supported by the Francis I. Proctor Foundation for Research in Ophthalmology of the University of California.

hospital patients selected at random, most of which were affected with neoplasms or heart disease, a 28% incidence of toxoplasmin sensitivity was demonstrated.

The high incidence of positive toxoplasmin reactors in patients with chorioretinitis is of especial interest. The mean age of these toxoplasmin positive patients when first seen was 23 years. The mean age when their disease was first noted was 13 years. For the toxoplasmin negative group of patients with chorioretinitis both dates were near 28 years of age. Both patients in Group 5 had chorioretinitis and nystagmus soon after birth. Only one of them, however, is markedly retarded mentally, and he has cerebral calcifications.

Groups 6, 7 and 8 refer to family members of 4 patients with toxoplasmosis proven by pathological examination at autopsy. From 2 patients the organism was also isolated (Group 4). However, the diagnosis in cases of Group 5 rested on clinical evidence, corroborated by serological data. The patient in Group 10, who was toxoplasmin positive, was 10 years of age. He did not show other clinical signs of toxoplasmosis.

(c) *Demonstration of toxoplasma antigen*

in ventricular fluid of infants with hydrocephalus. Fluids obtained from ventricular puncture were centrifuged. One-tenth cm³ of the supernatant was injected intradermally into normal guinea pigs and into guinea pigs with chronic latent toxoplasmosis. Ventricular fluid from 2 patients from whom toxoplasma was isolated, evoked after 24 hours a large area of erythema and induration in the infected guinea pigs. No reaction was present in the normal animals. Fluid from a patient with *Escherichia coli* meningitis evoked a slight area of erythema and induration in the toxoplasmic guinea pigs, but none in the normal controls. Ventricular fluids from 4 other hydrocephalic infants gave negative reactions in both groups of guinea pigs. Guinea pigs previously injected with human brain or cerebrospinal fluid may not be used, since they show marked reactions of dermal hypersensitivity on reinjection with such material.

Discussion. The high degree of specificity of the toxoplasmin reaction was initially ascertained in normal guinea pigs and in those with chronic, latent toxoplasmosis. These findings were supported by a high degree of correlation between skin hypersensitivity and

the presence of toxoplasma neutralizing antibodies in man. This specificity was further confirmed by the anamnestic appearance or rise of complement-fixing and neutralizing antibodies, following injection of the skin test antigen into individuals exhibiting dermal hypersensitivity. The high incidence of toxoplasmin sensitivity in patients with chorioretinitis, with and without associated cerebral calcifications, and in the mothers of such patients is significant.

The ease of performance of the skin test and the rapidity with which diagnostic readings appear make this test most useful in surveys. Equivocal results which are so frequently obtained with toxoplasma neutralizing antibody tests³ were of exceedingly rare occurrence with the use of the skin test. False positive skin tests were not encountered. The incidence of falsely negative skin tests was possibly 3%, however, the chance that neutralizing antibody test might have been falsely positive on some of these patients cannot be excluded at present. Hence the importance of performing on the same patient skin tests, neutralizing antibody tests, complement fixation tests and the new tissue culture tests with the patient's white blood cells,⁵ in the presence of toxoplasma antigen. Reading the skin test after 96 as well as 48 hours might substantially reduce the number of false negative skin tests.

New light has been shed on the incidence of asymptomatic past and possibly persistent toxoplasmosis in the general population. Whereas there may be differences in various regions of the country, the relatively low figures quoted previously^{6,7,8} might be largely

due to the use of the neutralizing antibody test, which is less sensitive than the skin test. The differences in age incidence point to the common occurrence of asymptomatic infection in adults. The incidence of toxoplasmin sensitivity in patients with chorioretinitis, observed to be 71% in my patients, is higher than previously reported.^{3,8} Toxoplasmin has been used in the treatment of active chorioretinitis, which was associated with skin test and serological evidence of toxoplasmosis. This will be discussed in another publication.

The use of ventricular fluid of infants with hydrocephalus is suggested as an aid in making or disproving a presumptive diagnosis of neonatal toxoplasmic encephalitis.

Summary. The preparation of toxoplasmin, a skin testing antigen made of toxoplasma has been described. Toxoplasmin evokes reactions of a delayed (tuberculin) type of hypersensitivity in certain patients. There is a high correlation between toxoplasmin hypersensitivity and the presence of toxoplasma neutralizing antibodies. The latter may be of very low titre and may not be unequivocally demonstrable until an anamnestic rise has occurred following the injection of the skin test dose. The ease of performance and the rapidity with which clear-cut results are obtained, make the toxoplasmin skin test the most useful single aid in the diagnosis of past or latent toxoplasmosis, wherever isolation of the causative organism is not feasible. A test based on dermal hypersensitivity has been described facilitating the differential diagnosis of neonatal hydrocephalus.

⁶ Sabin, A. B., *PROC. SOC. EXP. BIOL. AND MED.*, 1942, **51**, 6.

⁷ Callahan, W. P., Jr., *PROC. SOC. EXP. BIOL. AND MED.*, 1945, **59**, 68.

⁸ Johnson, L. V., *Arch. Ophthalm.*, 1946, **36**, 677.

⁵ Nantz, F. A., and Blatt, H., *Ann. Allergy*, 1947, **5**, 554.

Cancer Detection and Therapy. Affinity of Neoplastic, Embryonic, and Traumatized Tissues for Porphyrins and Metalloporphyrins.*

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In the course of studies on the cocarcinogenic action of porphyrins^{1,2} many of the mice that were injected with methylcholanthrene and porphyrins developed tumors. When these mice died or were sacrificed, it was noted that the porphyrin which had been injected intraperitoneally had accumulated and concentrated in the subcutaneous sarcomas to such a degree that the tumor had become red fluorescent while other normal tissues had not. A search of the literature revealed that other investigators³ had also observed the tendency for injected hematoporphyrin to accumulate in neoplastic tissues of rats. They had, however, only recorded this observation without further attempts to study or interpret this phenomenon. The affinity of porphyrins for neoplastic tissue was so striking that we have studied this in some detail. It was realized at the outset that if this affinity of neoplastic tissues for porphyrins extended also to metalloporphyrins and thus to radioactive metalloporphyrins, and proved to be generally true for all tumors, then this class of substances could be utilized to improve the existing methods of cancer detection and therapy.

The present report is based on observations made on 240 tumor bearing and 50 non-tumor bearing mice. The neoplasms studied included methylcholanthrene-induced tumors (spindle-cell fibrosarcomas and rhabdomyosarcomas), transplanted fibrosarcomas, spontaneous mammary carcinomas and transplanted mammary

adenocarcinomas (variable type) that developed in this laboratory. The observations on the first 30 mice were non-systematic and extended over a period of about 2 years. In the first systematic experiment the affinity of all available types of mouse neoplasms was tested for their ability to concentrate hematoporphyrin. Eighty mice were used in this experiment. Forty of these bore tumors of one of the types mentioned above. Twenty tumor-bearing and 20 non-tumor bearing mice were injected intraperitoneally with 1 mg of hematoporphyrin. Eight of these hematoporphyrin injected mice and 8 non-injected controls were sacrificed at 24 hour intervals to determine the rate of development of maximum concentration of hematoporphyrin in the tumors. Most of the porphyrin injected migrated to the tumor within a period of 24-48 hours. Some of the porphyrin was eliminated through the liver and appeared in the red fluorescent feces. The greater omentum became red fluorescent and remained so for several days. Lymph nodes also became red fluorescent in all mice. The other normal tissues do not concentrate the hematoporphyrin. Hematoporphyrin-injected mice with carcinomas or sarcomas when sacrificed at the end of 24, 48 or 72 hours exhibited brilliant red fluorescent tumors in contrast to the tumors of the controls.

These observations were repeated several times and in subsequent experiments it was established that the porphyrin concentration as indicated by the red fluorescence of the tumor remains high for 10 to 14 days but gradually decreases. If the tumors contained necrotic centers the concentrations of porphyrin was greatest in and near the necrotic areas but was also concentrated throughout the tumor and near the periphery. The spontaneous and transplanted carcinomas and the induced or the transplanted

*This work was supported by grants from the Anna Fuller Fund and the Gelatin Products Corporation.

¹ Figge, Frank H. J., *A.A.A.S. Research Conference on Cancer*, Science Press, 1945, 117-128.

² Figge, Frank H. J., *Ann. Int. Med.*, 1947, **27**, 143.

³ Auler, Hans, and Banzer, George, *Z. Krebsforsch.*, 1942, **53**, 65.

sarcomas—in other words all types of tumors tested—concentrated the hematoporphyrin. This indicated that the affinity of the tumors for porphyrin was not specific but perhaps general for all tumors.

Since regenerating and embryonic tissues are similar in some respects to neoplastic tissues, it was desirable to know whether these tissues would also concentrate porphyrins. The injection of hematoporphyrin into pregnant mice revealed that much of the porphyrin accumulated in the placentae and in the embryos. When hematoporphyrin was injected into mice which had been incised or otherwise traumatized, the porphyrin became concentrated at the site of injury and near the regenerating margins of incisions. These experiments indicated that growing tissues in general have an affinity for hematoporphyrin.

It is therefore probable that all neoplasms will have an affinity for porphyrins similar to that observed for this limited number of mouse tumors. It was known from the numerous observations in the experiments on co-carcinogenesis that neoplastic tissue had an affinity for both hemo and protoporphyrin but it was desirable to know whether this extended to other porphyrins. Mesoporphyrin and coproporphyrin were also tried and these substances also concentrated in neoplastic tissue. At the pH of the tissues the fluorescence intensity of protoporphyrin and coproporphyrin is not as great as hematoporphyrin and the concentrating effect does not seem as spectacular. A number of other fluorescent substances (fluorescein, rhodamine, dihydrocollidine and riboflavin) were also tested. None of these exhibited the same affinity for the tumors.

Perhaps the most important question was whether the neoplastic tissues would have

the same affinity for metalloporphyrins. Heme (iron-protoporphyrin) was tested intensively but no conclusions could be drawn because the heme does not fluoresce and therefore could not be distinguished easily from hemoglobin and other substances present in tumor tissues. Zinc hematoporphyrin was therefore prepared by one of us (G.W.) for the express purpose of testing the affinity of neoplastic tissue for metalloporphyrin. Zinc hematoporphyrin in contrast to heme and many other metalloporphyrins fluoresces with a characteristic fluorescence spectrum and can easily be traced through the bodies of mice. A repetition of the experiment described above using 10 mice bearing transplanted methylcholanthrene-induced sarcomas demonstrated that the zinc hematoporphyrin also accumulated in tumor tissues. Even though the fluorescence intensity of this compound is not as great as hematoporphyrin at the pH of the tissues it was possible to visually observe the fluorescence spectrum of this compound in tumor tissues whereas this could not be seen in adjacent normal tissues.

Summary and Conclusions. These experiments show that all porphyrins tested accumulate in neoplastic (induced and transplanted sarcomas, spontaneous and transplanted mammary carcinomas), embryonic and regenerating tissues. Introduction of a metal (zinc) into the porphyrin molecule did not destroy the tendency of the porphyrin to concentrate in tumors. The tendency of injected porphyrins and metalloporphyrins to accumulate in lymph nodes may limit the therapeutic usefulness of these compounds in all neoplastic diseases except lymphatic leukemias. The possibility of using small doses of radioactive metalloporphyrins for detecting deep cancer is being investigated.

Effect of Nicotinic Acid on Anoxia in Rats.

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On theoretical grounds, it is readily conceivable that the administration of nicotinic acid or its amide might be helpful, at least temporarily, in offsetting certain of the effects of reduced oxygen tension. As the prosthetic group of diphosphopyridine nucleotide, it has the capacity of accepting hydrogen from 3-phosphoglyceraldehyde, a triose resulting from the enzymatic breakdown of glucose. This transfer results in the release of energy at the expense of reduction of the coenzyme. Under ordinary aerobic conditions, the phosphoglyceric acid so produced undergoes enzymatic rearrangements to form pyruvic acid (von Euler *et al.*,¹) which is then oxidized by way of thiamine and the flavoprotein-cytochrome systems to carbon dioxide and water. At the same time, reduced coenzyme is reoxidized and, thus reactivated, may again participate in the primary oxidation of triose. (Reviewed by Ball²).

Under anaerobic conditions, on the other hand, the failure of reduced coenzyme to be reactivated results in reorientations of the oxidative pattern, varying apparently on the degree of reduction of oxygen tension. Ball states² that if the oxygen tension is so reduced that the energy demands of the cell per unit of time are not met, then pyruvic acid and reduced pyridine nucleotide interact to yield lactic acid and oxidized coenzyme, this process finally being halted either by equilibrium conditions or acid formation. Kempner³ has likewise shown that, as oxygen

tension is reduced below a certain critical level, cellular respiration declines in rate and also changes qualitatively. Barron⁴ is of the opinion that under such circumstances the release of energy depends on dehydrogenation rather than decarboxylation. These several lines of evidence are at variance with the earlier contentions of Meyerhof⁵ and Warburg⁶ that cellular respiration is entirely independent of oxygen tension and that it proceeds at a normal rate so long as the smallest amount of oxygen is present.

As corollary to the evidence just cited, it would appear that the enzymatic phases of cellular respiration might constitute a protective mechanism, useful in tiding the animal organism over a temporary period of reduced oxygen tension. This indeed may well be the device by which the tissues are enabled to accumulate an "oxygen debt" during excessive muscular exercise. Obviously, the effectiveness of this mechanism depends on an adequate supply of oxidized coenzyme; and if part of the latter be trapped in the reduced state because of lack of oxygen, then it is possible that the administration of nicotinic acid might augment the available supply of coenzyme and thus, in effect, correct this induced deficiency. Credence in this hy-

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Laboratory facilities were generously provided by the Department of Physiology and Pharmacology, School of Medicine, Duke University.

¹ von Euler, H., Adler, E., Günther, G., and Hellström, H., *Z. physiol. Chem.*, 1937, **245**, 217.

² Ball, E. G., *Bull. Johns Hopkins Hosp.*, 1939, **65**, 253.

³ Kempner, W., *Cellular and Comp. Physiol.*, 1937, **10**, 339; *Cold Spring Harbor Symposia on Quantitative Biology*, 1939, **7**, 269.

⁴ Barron, in discussion of Kempner (3-b).

⁵ Gildemeister, M., and others, editors, *Monographien aus dem Gesamtgebiet der Physiologie der Pflanzen und der Tiere*, Herausgegeben von M. Gildemeister, R. Goldschmidt, C. Neuberg, J. Parnas, und W. Ruhland. Band XXII: Die chemischen Vorgänge in Muskel und ihr Zusammenhang mit Arbeitsleistung und Wärmebildung, von Otto Meyerhof. Berlin, 1930, Julius Springer.

⁶ Warburg, O., and Kubowitz, F., *Biochem. Z.*, 1929, **214**, 4.

TABLE I.
Effect of Nicotinic Acid (or Its Amide) on Capacity of Rats to Survive at Low Barometric Pressures.

Pair No.	Untreated		Treated with nicotinic acid	
	Pressure at death, mm Hg.	Corresponding altitude, feet	Pressure at death, mm Hg.	Corresponding altitude, feet
1	80	52,000	60	58,000
2	80	52,000	60	58,000
3	100	47,000	60	58,000
4	80	52,000	40	66,000
5	80	52,000	80	52,000
6	80	52,000	60	58,000
7	80	52,000	60	58,000
8	80	52,000	60	58,000
9	100	47,000	80	52,000
10	80	52,000	80	52,000
11	100	47,000	100	47,000
12	100	47,000	100	47,000
13	100	47,000	80	52,000
14	80	52,000	60	58,000
15	100	47,000	100	47,000
16	100	47,000	100	47,000
17	200	32,500	80	52,000
18	80	52,000	80	52,000
19	80	52,000	80	52,000
20	80	52,000	80	52,000
Avg	93	49,275	75	53,800

pothesis is increased by observations of Kohn⁷ and of Axelrod *et al.*⁸ that the giving of nicotinic acid is followed by an increase in the concentration of the pyridine nucleotides. Decharneux⁹ showed that the diethylamide of nicotinic acid (coramine) offsets some of the effects of high altitude (Cheyne-Stokes respiration, convulsions, and eventual death); and Morell,¹⁰ noting an increase in the capacity for hard muscular exercise at sea level produced by nicotinamide, suggested that it might be beneficial in aviation.

In view of these theoretical considerations, it seemed worth while to investigate the effect of nicotinic acid on animals subjected to low barometric pressures. The results indicate

that the tachypnea associated with reduced oxygen tension is significantly alleviated and that animals so treated are probably enabled to survive equivalent altitudes that otherwise would be fatal.

Experimental. (a) *Effect of Nicotinic Acid (and its Amide) on Capacity of Rats to Survive at Increasingly Low Barometric Pressures.* Twenty pairs of rats of the Vanderbilt strain¹¹ were used, their weights averaging 125 g. Two pairs at a time were placed in a desiccator and the air evacuated at a rate to simulate an ascent of 750 feet per minute. This rate was chosen so as to avoid the cumulative effects of gradually induced anoxia. Ventilation was assured by means of a small intake valve, and the room in which the experiments were conducted was air-conditioned (T 72° F; relative humidity, 50%). Half of the treated group received 1 mg nicotinic acid intraperitoneally,

⁷ Kohn, H. I., *Biochem. J.*, 1938, **32**, 2075.

⁸ Axelrod, A. E., Gordon, E. S., and Elvehjem, C. A., *Am. J. Med. Sc.*, 1940, **199**, 697.

⁹ Decharneux, G., *C. R. Soc. de Biol.*, 1933, **112**, 692.

¹⁰ Morell, T., *Deut. med. Wchnschr.*, 1940, **66**, 398.

¹¹ Wolfe, J. M., Bryan, W. R., and Wright, A. W., *Am. J. Cancer*, 1938, **34**, 352.

TABLE II.
Effect of Nicotinic Acid (1 mg) on Respiratory Rate of Rats at Barometric Pressure of 300 mm Hg. (Group A).

Animal No.	Respiratory rate at 760 mm	Respiratory rate at 300 mm	Absolute increase in rate
Untreated:			
1	72	120	48
2	80	174	94
3	108	192	84
4	90	192	102
5	120	216	94
6	108	186	76
7	96	162	66
8	132	252	120
9	96	150	54
10	120	192	72
Avg	102.2	183.6	81 (79.3%)
Treated with Nicotinic Acid:			
1	96	108	12
2	102	126	24
3	174	240	66
4	96	132	36
5	90	108	18
6	120	156	36
7	108	144	36
8	120	132	12
9	132	108	(—) 24
10	120	156	36
Avg	115.8	141	25.2 (21.8%)

Statistical analysis: Fisher's *t* test, on absolute increases in respiratory rates, gives a *t* value of 5.4572; degrees of freedom, 18; *P* is less than 0.001. The results therefore are highly significant.

and the other half an equivalent amount of nicotinamide; the control animals were injected with identical volumes of physiologic saline solution. The last gasp of each animal was accepted as a criterion of death.

Results of this experiment are shown in Table I. Since the behavior of the animals receiving free nicotinic acid and those treated with nicotinamide was identical, the two groups have been pooled in this table. Respiration of the untreated animals ceased at an average pressure of 93 mm Hg (corresponding to an altitude of 49,275 feet), whereas the final respiratory movements of the treated animals occurred at an average pressure of 75 mm Hg (53,800 feet elevation).

Statistical analysis by Fisher's *t* test yields a *t* value of 2.5245; degrees of freedom, 38; *P* then lies between 0.01 and 0.02. The results are therefore probably significant. It will be noted that one of the untreated animals died at a pressure of 200 mm Hg,

while one of the treated animals survived until a pressure of 40 mm was reached. While the erratic behavior of these 2 animals unduly affects the average lethal pressures in the 2 groups, it does not, of course, affect the validity of the statistical conclusion.

(b) *Effect of Nicotinic Acid on Respiratory Rate of Rats at Low Barometric Pressures.* The animals in Group A, comprising 20 rats whose weights averaged 150 g, were subjected to a barometric pressure of 300 mm Hg attained gradually over a period of 30 minutes. In order to prevent violent fluctuations in pressure, a 5 gallon bottle was interposed between the vacuum pump and the desiccator holding the animals. Fresh air was admitted through a needle valve. Half of the animals were treated with 1 mg nicotinic acid in 0.5 cc diluent, and the other half received similar injections of physiologic saline solution. Respiratory excursions were recorded photographically by means of a differential pressure manometer designed by

TABLE III.
Effect of Nicotinic Acid Amide (5 mg) on Respiratory Rates of Rats at Barometric Pressures of 300 mm and 250 mm Hg. (Group B).

Animal No.	Respiratory rate at 760 mm	Respiratory rate at 300 mm	Increase	Respiratory rate at 250 mm	Increase
Controls:					
1	76	168	92	180	104
2	82	158	76	*	*
3	76	190	114	190	114
4	70	138	68	170	100
5	84	148	64	164	80
6	76	152	76	142	66
7	86	174	88	178	92
8	76	135	59	†	†
9	80	*	*	*	*
10	82	160	78	†	†
Avg	78.8	157.2	79.5 (100%)	172.3	93 (118%)
Treated:					
1	82	138	56	140	58
2	72	128	56	143	71
3	70	120	50	126	56
4	72	98	26	106	34
5	80	120	40	128	48
6	90	147	57	140	50
7	82	130	48	166	84
8	78	138	60	158	80
9	82	128	46	123	46
10	84	132	48	114	30
Avg	79.2	127.9	48.7 (61.5%)	134.9	55.7 (70.3%)

* Respiration failing; too irregular to be counted.

† Respiration ceased.

Statistical analysis: (1) Increases in rate at 300 mm Hg.: t , 4.8026; degrees of freedom, 17; P , less than 0.001. (2) At 250 mm Hg.: t , 4.015; degrees of freedom, 14; P , between 0.01 and 0.001. The differences in both sets of data are highly significant.

Hurst,¹² readings being taken before injection (at 760 mm), 10 minutes after injection (760 mm), and 40 minutes after injection (at a pressure of 300 mm Hg). In order to minimize psychic variants, all animals were trained by being placed in the apparatus daily for a week preceding the actual experiment.

The procedure in Group B differed from the above only in the following particulars: the animals averaged 225 g in weight; 5 mg nicotinamide was used instead of 1 mg nicotinic acid as in the first group; respirations were counted visually, as timed with a stopwatch; and observations were made both at 300 mm Hg and again at 250 mm.

Results of the experiment on Group A are shown in Table II. Since the rates before

injection and 10 minutes afterward did not differ significantly, the latter have been omitted from the table. The absolute increase in respiratory rates of the untreated animals was 81 per minute (79.3%), while that of the treated animals was 25.2 per minute (21.8%). Fisher's t test on the absolute increases in rate shows that the results are highly significant.

Data on the experiment with Group B are shown in Table III. At 300 mm pressure, the average increase in respiratory rate of the untreated animals was 79.5 (100.0%), against an average increase of 48.7 (61.5%) in the animals receiving nicotinamide; the corresponding increases at 250 mm were 93 (118.0%) and 55.7 (70.3%) respectively. As will be noted from the table, 2 of the untreated animals died at 250 mm pressure,

¹² Hurst, W., *Rev. Sci. Instruments*, 1941, **12**, 265.

and in several instances, both at 300 mm and at 250 mm, the respiratory movements of the untreated rats were too irregular to be counted with accuracy. This latter irregularity consisted of periodic breathing of the Cheyne-Stokes type, the periods of complete apnea being very long. The differences in respiratory rates of the contrasting groups, both at 300 mm and at 250 mm, are highly significant when analyzed by Fisher's *t* test.

Summary. Rats treated with nicotinic acid or its amide were found to survive until they reached a barometric pressure of 75 mm Hg (equivalent altitude, 53,800 feet); untreated

controls died at an average pressure of 93 mm Hg (49,275 feet). Statistically, the differences are probably significant.

The tachypnea developing at low barometric pressure is much less in treated than in untreated rats. Preliminary injection of 1 mg nicotinic acid was followed by an increase in respiratory rate of only 22% at 300 mm Hg as opposed to approximately 80% in untreated controls. Differences of comparable magnitude were noted at pressures of 300 and 250 mm Hg following the administration of nicotinamide. Statistical analyses indicate that these differences are highly significant.

16582

Effect of 3,3'-Methylene-Bis-4-Hydroxycoumarin (Dicumarol) on Embryonic Chick Liver and Spleen.*

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Dicumarol is being used today in the control and possible prevention of thrombo-embolic phenomena. Its mode of action is as yet not well understood. Some workers have presumed that it works by inhibiting the utilization of vitamin K by the liver cell in the synthesis of prothrombin. Others think that it may act as a toxic agent on the liver cells, especially when used in large amounts.¹ This study was initiated to observe the effect of dicumarol on isolated sections of liver and spleen of the 14 day chick embryo.

Method. 14 day chick embryos were removed from their shells and placed in petri dishes containing Tyrode's solution. The liver and spleen were isolated and small portions were placed in individual petri dishes containing various concentrations of dicumarol in a 10% rabbit serum-Tyrode solution. Since the volume of the medium used was small, the pieces of tissue were sectioned in the petri

dishes containing the dicumarol in the given concentrations. This was done in order to prevent dilution of the dicumarol serum-Tyrode medium. These pieces of liver and spleen were then cut into approximately one cubic millimeter sections with a sharp scalpel and placed on 20 x 20 mm No. 2 cover slips in a small drop of media containing the disodium salt of dicumarol in 10% rabbit serum and Tyrode's solution. These cultures were placed on deep-welled micro culture slides in a lying-drop position, sealed with paraffin and incubated at 38° C. The disodium salt was prepared by adding the crystalline material to .345 N sodium hydroxide. The same amount of base was added to the media containing the different amounts of dicumarol. The pH of the media ranged from 7.74 to 7.88. Little or no proliferation of cells was noted at 24 hours. This was especially true in the liver cultures. Therefore, the period of observation was begun at 48 hours. The longest period of observation was 72 hours. According to Nordman, before 14 days an extensive proliferation of

* This study was supported by a grant-in-aid from the United States Public Health Service.

¹ Rose, C. L., Harris, P. N., and Chen, K. K., *Proc. Soc. Exp. Biol. and Med.*, 1942, **50**, 228.

TABLE I.
Effect of Dicumarol on Growth of Embryonic Chick Liver and Spleen in Tissue Culture.

Dilution	Period of observation (hr)	Liver		Spleen	
		No. of cultures	Proliferating cells	No. of cultures	Proliferating cells
Control	48	11	+	8	+
1/10,000		5	+	3	+
1/50,000		4	+	1	+
1/100,000		5	+	3	+
Control	72	8	+	3	+
1/10,000		5	0	3	+
1/50,000		4	+	1	+
1/100,000		5	+	3	+

fibroblasts is not observed in the embryonic liver.² The appearance and growth pattern of these cells indicates that they are proliferating endothelial cells. The proliferating cells of the spleen cultures were predominantly blood elements. Those seen in the media were immature and mature polymorphonuclear leucocytes and nucleated red cells.

At the end of these periods of observation the cover slip containing the liver tissue was removed and placed in a small stendor dish containing Bouin's solution. After one hour in Bouin's solution the small pieces of tissue, which remained very firmly fixed to the cover slips, were washed in tap water for one hour, in a saturated aqueous solution of lithium carbonate for fifteen minutes, in tap water again for 15 minutes, and in 3 changes of distilled water. They were then stained in Harris's haematoxylin, dehydrated, and mounted in clarite on micro slides.

A number of the spleen cultures were fixed in the above manner. Some, however, were allowed to dry and were stained with Wright's solution.

Results. All the control cultures of liver show proliferation of endothelial-like cells at

48 and at 72 hours. In concentrations of dicumarol of 1/50,000 and 1/100,000, the cultures contained living cells at the end of 48 as well as 72 hours as evidenced by cell structure and staining reactions after fixation. Those cultures containing dicumarol in concentrations of 1/10,000 showed proliferating cells at the end of 48 hours, but in all of these cultures incubated for 72 hours the cells died and disintegrated. Spleen cultures of 14 day chick embryos showed cellular proliferation of blood elements in media containing dicumarol in concentrations of 1/50,000 and 1/10,000 at 48 and 72 hours.

Summary. The effect of the disodium salt of dicumarol was observed on 14 day embryonic chick liver and spleen cultured in a 10% rabbit serum and Tyrode solution. Concentrations of dicumarol of 1/100,000 and 1/50,000 did not appear to inhibit the proliferation of endothelial cells at 48 and 72 hours when compared with control cultures. However, with a concentration of 1/10,000 cellular proliferation was present at 48 hours, but at 72 hours these cells had been killed and lysed. In contrast, spleen cultures of 14 day chick embryos showed cellular proliferation in media containing dicumarol in concentrations of 1/50,000 and 1/10,000 at both 48 and 72 hours.

² Nordman, M., *Arch. f. exp. Zellforsch.*, 1929, 8, 371.

Effect of Size of Inoculum on the Apparent Vitamin Requirements of Lactic Acid Bacteria.*

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The vitamin requirements of 23 lactic acid bacteria, as reported recently by Shankman, Camien, Block, Merrifield and Dunn,¹ were, in many cases, contrary to those previously reported. For example, biotin, nicotinic acid and pantothenic acid, which have previously been found essential for all lactic acid bacteria tested^{2,3,4} were reported as unessential for growth of several organisms, including *Lactobacillus arabinosus* and *Lactobacillus casei*. These findings were especially unexpected inasmuch as the latter two organisms have been widely used for determination of each of these vitamins. These authors attributed their results to use of an exceptionally complete basal medium, which permitted synthesis (though limited, in some cases) of vitamins required for growth in less complete media.

An investigation to determine the cause for these variable findings was undertaken. In their experiments, Shankman, *et al.* used an exceptionally heavy inoculum, which had been grown in a medium rich in vitamins. It was considered possible, therefore, that their results were complicated in some cases by carry-over of sufficient vitamin with the inoculum to permit growth.

Experimental. A series of tests was made in a medium⁵ similar to those used for vitamin

assay, which contained hydrolyzed casein as the nitrogen source. The inoculum was grown in 10 ml of the complete medium. After 18 hours, cells were centrifuged, resuspended in 30 ml of 0.9% sodium chloride solution, and 1 ml of this suspension was used to inoculate 10 ml of the basal medium which lacked the vitamin under investigation. This inoculum is similar to that used by Shankman *et al.*¹ Additional series were inoculated with 1 ml of 1:7, 1:70, or 1:700 dilutions of this inoculum. These 4 inocula are referred to as inoculum A, B, C and D, respectively. Turbidity was measured at one or 2 day intervals over a period of 7 days. Typical data are given in Table I. In the complete medium, supplemented with all essential vitamins, heavy growth was obtained with all inocula. In the nicotinic acid and pantothenic acid-deficient media, inoculum A permitted heavy growth; inoculum B much lighter growth, and inocula C and D essentially no growth. These data indicated that growth in vitamin-deficient media was due to introduction with the heavy, washed inoculum of sufficient quantities of vitamin to permit heavy growth. An alternative explanation would be that only a few cells capable of vitamin-synthesis were present among the population of the inoculum culture, and that with heavy inocula, but not with light inocula, enough of these were transferred to the deficient medium to initiate growth.

It is possible to decide between these two explanations by subculture. If the cells which grow in the deficient medium synthesize their own vitamins, they should continue to grow in the deficient medium on subculture. If on the other hand, growth in the deficient medium results from carry-over of vitamins with

* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by a grant from the Research Grants Division, National Institute of Health, U. S. Public Health Service.

¹ Shankman, S., Camien, M. N., Block, H., Merrifield, R. B., and Dunn, M. S., *J. Biol. Chem.*, 1947, **168**, 23.

² Snell, E. E., *J. Bact.*, 1945, **50**, 373.

³ Peterson, W. H., and Peterson, M. S., *Bact. Rev.*, 1945, **9**, 49.

⁴ Knight, B. C. J. G., in Harris, R. S., and Thimann, K. V., *Vitamins and Hormones*, New York, 1945, **3**, 106.

⁵ Rabinowitz, J. C., Mondy, N. I., and Snell, E. E., *J. Biol. Chem.*, 1948, **175**, 147.

TABLE I.
Effect of Size of Inoculum on Growth of *Lactobacillus arabinosus* in Vitamin-Deficient Media.

Medium	% of incident light transmitted*							
	Inoculum A		Inoculum B (1:7 dilution of A)		Inoculum C (1:70 dilution of A)		Inoculum D (1:700 dilution of A)	
	24 hr	168 hr	24 hr	168 hr	24 hr	168 hr	24 hr	168 hr
Nicotinic acid deficient	53	38	84	83	92	90	93	90
Pantothenic acid deficient	53	38	88	82	93	91	95	95
Complete	20	17	23	18	32	20	72	17

* Distilled water = 100.

TABLE II.
Growth in Subcultures from Vitamin-Deficient Media.

Medium	% of incident light transmitted*							
	<i>L. arabinosus</i>				<i>L. casei</i>			
	Original		Subculture I		Original		Subculture I	
	24 hr	168 hr	24 hr	168 hr	24 hr	168 hr	24 hr	168 hr
Nicotinic acid deficient	53	40	90	87	53	40	91	91
Complete	18	16	16	14	32	21	44	25
Pantothenic acid deficient	46	46	90	87	57	51	92	92
Complete	19	20	19	19	32	21	30	23
Biotin deficient	36	35	91	91	56	40	91	92
Complete	19	20	18	18	32	21	39	22

* Uninoculated medium = 100.

the inoculum, this should not be sufficient to permit growth in the deficient medium on repeated subculture. In testing this procedure, the inoculum medium, the size of the inoculum, and the basal media used were those described by Shankman, *et al.*¹ Ten ml volumes were used and turbidities were read at 24, 48, 72 and 168 hours. In confirmation of the results of Shankman and coworkers,¹ heavy growth of both organisms occurred in media free of pantothenic acid, nicotinic acid, or biotin (Table II). When heavy growth was obtained in these media, 1.0 ml was transferred to 10 ml of similarly deficient media, and to the same medium with all vitamins added. In the subcultures, no growth occurred without the vitamins, although good growth occurred when these were present (Table II). These results demonstrate that these organisms do not synthesize biotin, pantothenic acid or nicotinic acid in amounts sufficient to permit significant growth under

these conditions. They do not, of course, deny the possibility that cultures of these organisms might be obtained by adaptation or selection which would grow in the absence of these vitamins. It is not necessary, however, to postulate this occurrence to explain growth in vitamin-free media when heavy inocula, grown in vitamin-rich media, are employed.

Discussion. When grown in the presence of an excess of certain vitamins, several microorganisms are known to accumulate amounts in the cell far higher than those present when cells are grown with only sufficient of the vitamin to permit maximum growth. *Lactobacillus pentosus*, for example, accumulates up to 15 times as much biotin in its cells when grown with excess biotin as it does when grown with minimal amounts of biotin.⁶ Since the latter quanti-

⁶ Krueger, K. K., and Peterson, W. H., *J. Bact.*, 1948, **55**, 693.

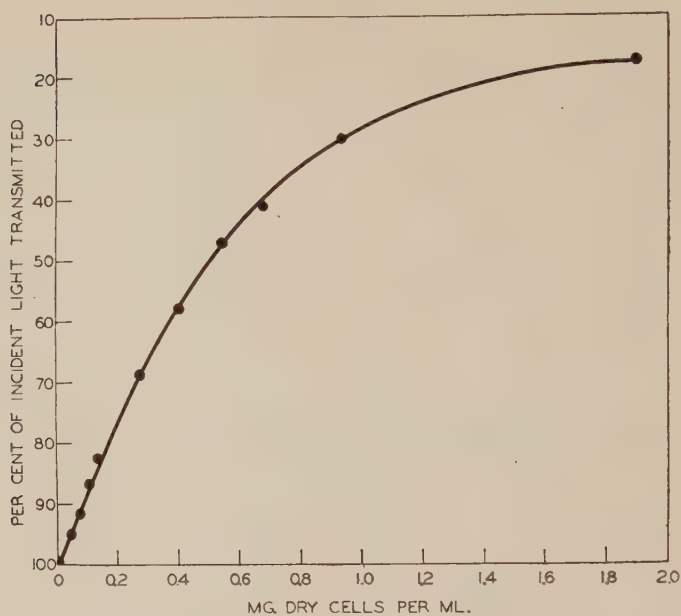


FIG. 1.

The relationship between culture turbidity and weight of cells for *Lactobacillus arabinosus*.

ties are sufficient to permit heavy growth, it would be expected that biotin-requiring cells containing a 15-fold excess over their minimum requirements of biotin would be able to increase in mass as much as 15 times when inoculated into a biotin-free medium before growth would cease for lack of biotin. If the inoculum is sufficiently heavy, increases of this magnitude will appear as heavy growth; with lighter inocula, a similar increase would not suffice to bring the number of cells into the visible range. These relationships are clearly apparent from Fig. 1, which relates light transmission to weight of cells of *Lactobacillus arabinosus* under our conditions. Inoculum A in 10 ml of medium gives a reading of 90, corresponding to .08 mg of dry cells per ml which lend a barely visible turbidity to the medium. A 15-fold increase in mass, which can occur without added biotin, would give 1.2 mg of cells per ml, which appears heavily turbid (galvanometer reading 24). A 70-fold dilution of this inoculum might also increase 15 times in mass, but would still give

less than 0.02 mg of cells per ml, an amount too small to be detected visually.

These results emphasize the extent to which experimental results with certain microbiological methods may differ depending upon the size and history of the inoculum. Lack of appreciation of these simple relationships has been the source of very considerable difficulty in applying microbiological methods for vitamin assay.

Summary. Discrepancies in the findings of various investigators relative to the requirement of certain lactic acid bacteria for pantothenic acid, nicotinic acid, and biotin have been resolved. Recognition of the essential nature of these and other vitamins may be prevented by use of heavy inocula, from cultures grown in vitamin-rich media. This appears due to the fact that some organisms store certain vitamins in excess of their requirements, and thus are able to grow to a limited extent when subsequently transferred to media free of the essential vitamin or growth factor.

Production of Positive Inotropism in the Dog Heart by Some Constituent of Intocostrin.*

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Recent clinical and laboratory work in connection with curare as an adjuvant to anesthesia has disclosed little significant action of this drug on the myocardium. Older experimental work is rather inconclusive because of the impure preparations used. Carlson¹ reported that in the *Limulus* heart curare produces an inhibitory effect by acting on the heart ganglion but that it does not affect the heart muscle or the intrinsic motor nerve fibers. Jullien² found a negative inotropic effect on the oyster heart. Hauschild³ stated that curin has a negative inotropic, chronotropic, and dromotropic effect on the frog heart, but Tillie⁴ described the action of curin on the frog heart as being like that of veratrin or glucosides of the digitalis group. It is the purpose of this paper to report the effects produced by certain curare preparations in the isolated dog heart.

Method. The dog heart-lung preparation was used in these studies and was arranged for the recording of right atrial pressure, aortic pressure, pulmonary arterial pressure, and cardiac output.[†] Ventricular volume was recorded mechanically upon smoked kymograph paper by means of a Henderson cardiometer and a piston-type volume recorder. No alterations were made in venous return or the peripheral resistance during the course of an observation, and the curare preparations were

introduced directly into the venous return.

Two curare preparations were used: *d*-tubocurarine chloride (Squibb) and a less highly purified curare preparation, Intocostrin (Squibb). It should be noted that according to the manufacturer, 50% of Intocostrin is *d*-tubocurarine and that the *d*-tubocurarine content accounts for all of its paralytic activity. The remainder of the Intocostrin is unidentified residue from *Chondrodendron tomentosum*.

Results. Intocostrin was used 15 times in 10 different heart-lung preparations in amounts above 5 units and in every instance there was a prompt reduction in both systolic and diastolic heart size without a change in cardiac rate. In one case there was a slight fall in aortic pressure and cardiac output. When the effect was most marked there was a transient rise in aortic pressure associated with the momentary increase in cardiac output. The reduction in heart size was not permanent, the heart gradually returning to its normal volume. Maximal effects were obtained with about 20 units, larger amounts producing little additional effects. This effect was obtained both in fresh heart-lung preparations and in those in which some degree of heart failure was present, seeming to be more striking when failure was present.

The pure drug *d*-tubocurarine chloride was introduced at a comparable dose level 7 times in 5 different heart-lung preparations and in each instance it was without any effect upon the observed pressures, the cardiac output, the cardiac rate, or the heart size, except in one case in which it produced a slight increase in heart size. The preservative, chlorobutanol, was used in amounts identical with those amounts present in the Intocostrin solution, and it likewise was without effect. In Fig. 1

* Aided by grants from the Research Board of the University of California.

¹ Carlson, A. J., *J. Gen. Physiol.*, 1894, **34**, 147.

² Jullien, A., *C. R. Soc. de Biol.*, 1936, **121**, 1002.

³ Hauschild, F., *Arch. f. exp. Path. u. Pharmacol.*, 1934, **174**, 742.

⁴ Tillie, J., *Arch. f. exp. Path. u. Pharmacol.*, 1890, **27**, 1.

[†] The experimental procedure used is described in greater detail elsewhere (*J. Pharm. and Exp. Therap.*, in press).

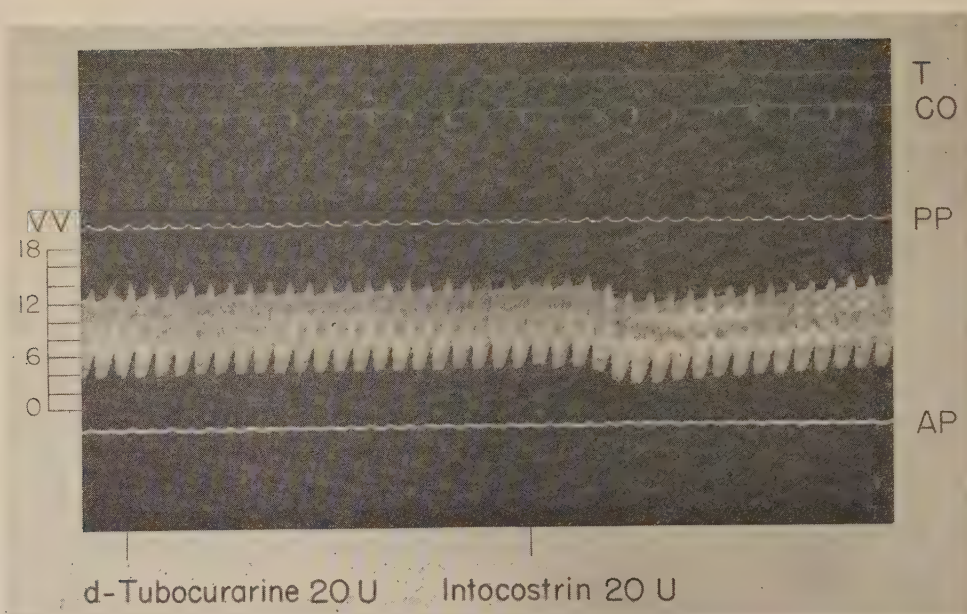


Fig. 1.

T, time in minutes; CO, cardiac output; PP, pulmonary arterial pressure; AP, aortic pressure; VV, ventricular volume change calibrated in cc; diastolic size is indicated by the upper part of the stroke, systolic size by the lower.

TABLE I.
Effect of Curare upon Aortic Pressure, Cardiac, Output, and Ventricular Volume of the Dog Heart-lung Preparation.

Curare preparation used and dose	Effect on aortic pressure	Effect on cardiac output	Effect on ventricular volume
<i>d</i> -T* 5 μ	None	None	None
<i>d</i> -T 20 μ (5 experiments)	"	"	"
<i>d</i> -T 20 μ Int.† 5 μ (2 experiments)	"	"	1/2 cc increase None
Int. 20 μ (2 experiments)	"	"	1 1/2 cc decrease each
Int. 20 μ (2 experiments)	"	"	2 " " "
Int. 20 μ (2 experiments)	"	"	3 " " "
Int. 20 μ (2 experiments)	"	"	4 " " "
Int. 20 μ (2 experiments)	Transient rise of 4-6 mm of Hg.	"	4 " " each
Int. 20 μ	Transient rise of 10 mm of Hg.	Transient rise‡	10 " "
Int. 10 μ § 1 min later 10 μ	None	None	1 1/2 " "
1 min later 80 μ	Transient fall of 6 mm of Hg.	Transient fall‡	3 " "
4 min later 100 μ	Transient rise of 4 mm of Hg.	Not determined	5 " "
3 min later 100 μ	None	" "	1 " "

* *d*-tubocurarine chloride.

† Intocostarin.

‡ Because of the nature of the recording apparatus, transient changes could not be quantitated.

§ All subsequent administrations were to the same heart-lung preparation at time intervals as indicated. After the 80 μ dose and subsequently, there was partial recovery before the next administration.

is shown a representative kymograph tracing from the series of experiments illustrating the contrast between the two preparations. In Table I is presented a summary of the several experiments.

The average apnic dose for a dog is approximately 20 μ while 200 μ will produce apnea in about 80% of normal humans. Thus, the dose levels of Intocostrin producing significant effects in the dog heart-lung preparation are, in terms of units per mass of tissue,

considerably in excess of the amounts required for the desired paralyzing action in intact animals or man.

Conclusion. A highly purified curare preparation (Intocostrin) produces a positive inotropic effect in the isolated dog heart. This effect is not due to the *d*-tubocurarine content of the preparation or to the preservative and, therefore, must be due to some unidentified component.

16585

Antibiotic Studies on Beta Hemolytic Streptococci.* V. Streptomycin Resistance Acquired by Group A, B, and C Organisms.†

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The development of *in vitro* resistance to streptomycin has been reported for a number of organisms including staphylococci, meningococci, gram negative enteric bacilli, chromogenic bacteria, Klebsiella, and *Hemophilus influenzae*. Miller and Bohnhoff,¹ for example, demonstrated approximately a 2000 fold rise in resistance in four strains of gonococcus and nine of meningococcus after 4 to 6 daily transfers on streptomycin medium. Seligmann and Wassermann² described a 50,000 fold increase in streptomycin resistance by a strain of *Staphylococcus aureus* after 7 transfers on antibiotic medium and a change in excess of 50,000-fold in one strain of *B. prodigiosus* after 9 transfers.

Resistance by streptococci has not been thoroughly studied. In a group A, type 14 organism reported by Chandler and Schoen-

bach,³ a 5-fold rise in resistance after 6 daily transfers on streptomycin medium was observed. No other reference to streptomycin resistance of beta hemolytic streptococci has been found.

The present study compares the rate at which resistance develops among strains of Lancefield group A, B, and C streptococci. In addition we have attempted to compare the hemolytic, antigenic and virulence behavior of streptomycin resistant strains with that of penicillin resistant strains described in preceding communications.^{4,5,6}

The streptomycin sensitivity range in our laboratory⁷ for recently isolated strains of beta hemolytic streptococci has been found to be 10 to 50 $\mu\text{g/ml}$ for 275 strains of group A, 30 to 400 $\mu\text{g/ml}$ for 20 strains of group B, and 10 to 50 $\mu\text{g/ml}$ for 47 strains of group C

* These studies were partially supported by the Fenger Memorial Fund of the University of Chicago.

† This report was presented in part at the meeting of the American Association for the Advancement of Science in Chicago, December 30, 1947.

¹ Miller, C. P., and Bohnhoff, M., *J. Am. Med. Assn.*, 1946, **130**, 485.

² Seligman, E., and Wassermann, M., *J. Immunol.*, 1947, **57**, 351.

³ Chandler, C. A., and Schoenbach, E. B., *Proc. Soc. Exp. Biol. and Med.*, 1947, **64**, 208.

⁴ Gezon, H. M., *Proc. Soc. Exp. Biol. and Med.*, 1948, **67**, 208.

⁵ Gezon, H. M., *Proc. Soc. Exp. Biol. and Med.*, 1948, **67**, 212.

⁶ Gezon, H. M., *Proc. Soc. Exp. Biol. and Med.*, 1948, **67**, 215.

⁷ Unpublished data.

TABLE I.
Streptomycin Resistance Induced in Groups A, B, and C Hemolytic Streptococci by Subcultivation on Streptomycin Medium.

Group	Strain	No. of transfers	Initial streptomycin sensitivity,* $\mu\text{g/ml}$	Final streptomycin sensitivity,* $\mu\text{g/ml}$	Fold change
A	S ₁	40	25	3,500	140
A	S ₂	40	50	3,500	70
A	S ₃	40	10	1,400	140
A	S ₄	40	25	3,500	140
A	S ₅	40	25	6,000	240
A	S ₆	40	25	4,500	180
A	U ₅	40	50	3,500	70
A	U ₆	40	10	1,200	120
B	T ₅	24	75	30,000	400
B	T ₇	40	75	30,000	400
B	T ₈	40	100	4,000	40
B	T ₉	40	75	7,000	93
B	T ₁₀	40	75	12,000	160
B	T ₁₁	40	75	7,000	93
B	T ₁₂	24	100	30,000	300
C	U ₁	38	10	30,000	3,000
C	U ₂	40	25	15,000	600
C	U ₃	40	25	5,000	200
C	U ₇	40	50	12,000	240
C	U ₈	40	25	12,000	480
C	U ₉	40	25	5,000	200
C	U ₁₀	40	10	2,000	250
C	U ₁₁	40	25	6,000	240

* Streptomycin sensitivity is defined as the highest concentration of streptomycin in the medium which permits colonies to grow.

organisms. The Fleming⁸ ditch plate method was employed for these determinations. Sensitivities falling within these ranges were reported by Hobby and Lenert⁹ for two strains of group A and one each of groups B and C streptococci.

Materials and Methods. Twenty-three strains of beta hemolytic streptococci, 8 each of group A and C and 7 of group B, were isolated from cultures of the nose or throat obtained from patients with acute upper respiratory disease. None had received streptomycin therapy. The organisms were grouped by the Lancefield technic. The strain designations as well as many of the parent strains are the same as those used in a preceding communication.⁴

Bacto heart infusion agar (Difco) containing 5% defibrinated sheep's blood and varying concentrations of streptomycin was used in 100 mm Petri dishes. All plates were poured

at a maximal temperature of 50°C. Commercial streptomycin sulfate (Lilly), lot No. 6329-451634 with an expiration date of November 30, 1948, was employed for the entire study. Fresh stock solutions were made in normal saline on alternate days and refrigerated at 4°C. The potency of the streptomycin solutions was controlled by standardization against *Staphylococcus aureus* SM.

The ditch plates used to determine streptomycin sensitivity have been described previously.⁴

To induce resistance 7 or 8 strains of streptococci were inoculated in single lines on each blood agar plate. The group A, B, and C organisms were each considered as a separate series. Between 4 and 8 concentrations of streptomycin were used in a set for each serial transfer. The confluent bacterial growth on the blood agar plate was emulsified in saline and subcultured directly onto a new set of plates; a total of 40 such subcultivations was made in each series. Transfers to the next set of plates were made from colonies on medium with the highest concentration of

⁸ Fleming, A., *Proc. Roy. Soc. Med.*, 1941, **34**, 342.

⁹ Hobby, G. L., and Lenert, T. F., *Proc. Soc. Exp. Biol. and Med.*, 1947, **65**, 242.

TABLE II.
Reduction in Streptomycin Resistance After Passage Through Normal Mice.

Group	Strain	No. of transfers on streptomycin medium	<i>In vitro</i> streptomycin sensitivity, $\mu\text{g/ml}$	No. of passages in normal mice	Final streptomycin sensitivity after serial passages in normal mice, $\mu\text{g/ml}$
A	S ₁	40	3,500	15	1,000
A	U ₅	40	3,500	15	1,000
B	T ₅	24	30,000	15	4,000
B	T ₉	40	7,000	15	1,000
C	U ₁	38	30,000	15	20,000
C	U ₂	40	15,000	15	15,000

streptomycin permitting growth. The streptomycin sensitivity for each was considered to be the highest quantity of the antibiotic that allowed good growth. Serial transfers were discontinued when a strain had acquired a resistance of 30,000 μg of streptomycin per ml of medium.

The method for determining virulence by intracerebral inoculation of mice has been described in a preceding report.⁴ Streptolysin O and S production were determined by the methods given by Todd.¹⁰ Streptokinase (fibrinolysin) content was measured by the method outlined by Boisvert.¹¹

Results. A. Resistance Acquired on Medium Containing Streptomycin. Eight strains each of group A and C streptococci and 7 of group B were subcultivated serially in an attempt to induce resistance to streptomycin. Table I summarizes these results. The minimum increase in resistance was 40-fold in a group B organism and the maximum, 3000-fold in a group C organism, after 40 and 38 transfers respectively. In general the group A, B, and C organisms acted similarly, most strains showing from a 100- to 400-fold rise in resistance after 40 transfers on increasing concentrations of streptomycin. When the strains within a group are compared, the group C organisms are the most uniform in their rate of development of resistance and the group B the most variable.

B. Resistance Acquired on Control Medium. All 23 organisms were subcultured serially on control medium, *i.e.*, streptomycin-free blood agar. No increase in streptomycin resistance was found after 40 transfers, when

the organisms were tested by the streptomycin whole-plate technic or by the ditch plate method. No changes in hemolysis or in the colonial form were observed; a minimum of dissociation from mucoid to matt or matt to glossy was seen.

C. Loss of Acquired Resistance. Three strains each of group A, B, and C streptococci after acquiring streptomycin resistance were subcultured serially 100 times on streptomycin-free medium in an attempt to restore the original sensitivity. The organisms were tested on the 25th, 50th, and 100th transfer by the whole-plate method. No change in resistance outside the limit of experimental error was observed.

Two strains each of groups A, B, and C streptococci after acquiring streptomycin resistance were passed intracerebrally 15 times in normal mice. The results are given in Table II. Both strains of group A and both of group B demonstrated a significant loss of streptomycin resistance. One strain of group C (U₂) showed no change, and one a 33% reduction in resistance. None, however, returned to the original level of sensitivity.

D. Change in Virulence. Mouse virulence titrations were performed on parent strains, on subcultures of the same organisms after 40 transfers on control medium, and on the strain after 40 transfers on streptomycin medium. A total of 9 strains, 3 each of groups A, B, and C, were studied. The results are summarized in Table III. All of the group A and B streptococci showed a marked loss of mouse virulence after serial transfers on streptomycin medium. Two strains of group C showed a similar loss while one (U₂) remained unchanged. Most of the micro-

¹⁰ Todd, E. W., *J. Path. and Bact.*, 1938, **47**, 423.

¹¹ Boisvert, P. L., *J. Clin. Invest.*, 1940, **19**, 65.

TABLE III.
Mouse Virulence of Groups A, B, and C Streptococci with Acquired Streptomycin Resistance.

Group	Strain	Initial LD ₅₀ *	LD ₅₀ * after 40 transfers		LD ₅₀ * of resistant strains after 15 mouse passages
			Control medium	Streptomycin medium	
A	S ₁	.000024	.00060	>.03	.00030
A	S ₃	.00077	.00048	.0095	
A	U ₅	.000095	.00017	.0017	.00053
B	T ₅	<.0000030	.0000012	.030†	.00030
B	T ₈	<.0000030	.0000060	.0095	
B	T ₉	.00030	.00011	.0053	.0095
C	U ₁	.000095	.000060	.030‡	.0095
C	U ₂	.00060	.0017	.00053	.00095
C	U ₃	.00018	.000060	.0030	

* LD₅₀ is stated as the amount of undiluted bacterial suspension in normal saline that will kill 50% of the mice injected. This suspension has a density equal to McFarland No. 1 standard and contains approximately 2×10^8 organisms per ml.

† After 24 transfers.

‡ After 38 transfers.

organisms demonstrated relatively little loss of mouse virulence after serial transfers on control medium.

In an attempt to restore the virulence lost by growth on streptomycin medium, six strains were passed intracerebrally 15 times in normal mice. The virulence was only partially restored in 5 strains, and was unchanged in one (U₂) in which the original virulence had not been lost.

Since mouse virulence of beta hemolytic streptococci is probably related to at least two factors, M substance and hyaluronic acid, an effort is being made to demonstrate changes in these substances. Most investigators agree that hyaluronic acid is contained in the capsule of groups A and C streptococci. Using the India ink wet preparations described by Hirst and Lancefield,¹² we have looked for the presence or absence of a demonstrable capsule in 4-hour subcultures of parent and resistant organisms. There appears to be a loss of the capsule in both group A and C resistant bacteria. Confirmation of these observations is being sought through chemical studies.

E. Antigenic, Hemolytic, and Colonial Changes. All 23 organisms were again grouped by the Lancefield technic after acquiring streptomycin resistance. All remained group specific.

Studies are in progress on changes in streptokinase, streptolysin O, and streptolysin S production of representative parent and resistant streptococci. These will be reported in detail in the near future. A brief summary of the preliminary findings follows. None of the group B strains produced streptokinase or streptolysin O or S. All of the parent group A and C organisms and all except 2 of the resistant strains lysed a normal human fibrin clot in less than one hour. Two group A resistant strains failed to lyse the clot in 24 hours. Quantitative studies using purified human fibrinogen and human thrombin are being carried out.

Qualitative differences between parent and resistant organisms in streptolysin S production were found in the 2 group A and 3 group C streptococci studied. This difference will be quantitatively titrated against standard anti-streptolysin rabbit serum.

A change in the hemolytic behavior of streptococci when growing on streptomycin medium was observed one or more times in all 23 strains. Transient conversion from beta to alpha or gamma type of colonies appeared when the organisms were growing on maximal concentrations of streptomycin. The same strains showed the normal beta hemolysis on lower concentrations of streptomycin or on plain blood agar. In some instances a mixture of alpha, beta, and gamma types of colonies were evident on the same concentra-

¹² Hirst, G. K., and Lancefield, R. C., *J. Exp. Med.*, 1939, **69**, 425.

tion of streptomycin. Group C organisms showed this hemolytic change earliest and most consistently. None, however, demonstrated an irreversible change.

Minute colonies resembling glossy variants were observed with all strains when growing on maximal concentrations of streptomycin. These were not true glossy variants, however, since the usual matt form was seen in the next subcultivation on lower concentrations of streptomycin.

Discussion. Streptococci of groups A, B, and C develop streptomycin resistance at about the same rate and to the same degree. The relative rate of development of resistance to penicillin, in which group A organisms acquired resistance slowly and to a slight degree, group C intermediately, and group B most rapidly, has been previously described.^{4,5,6} When compared with resistance in meningococci,¹ or staphylococci,² streptococci acquire resistance relatively slowly.

The loss of virulence found in both penicillin- and streptomycin-resistant streptococci is of considerable interest. A simple explanation could be the concurrent loss of capsular substance with its content of hyaluronic acid. It has been fairly well demonstrated that this is at least one of the factors responsible for the virulence of the streptococcus. This is probably only a partial explanation. Further studies are being made on the type-specific M substance to determine whether a similar alteration occurs in this antigen, which seems to play an important role in the virulence of these organisms.

Another fundamental change in their bacterial metabolism is found in penicillin-resistant,^{4,5,6} streptomycin-resistant, and bacitracin-resistant¹³ streptococci; with all 3 antibiotics transient reversible changes in hemolysis from beta to alpha or gamma types of colonies were observed. After observing this phenomenon in cultures in our laboratory, Dr. Edgar W. Todd suggested that the change probably was in streptolysin S production of resistant organisms, since streptolysin S is largely responsible for surface hemolysis. We have studied only 5 strains of group A and C

streptococci to date but in all 5 the parent strains produced streptolysin S in large amounts and the resistant members produced little or none. Final proof of this depends, however, on titration of these lysins against a known anti-streptolysin S serum. This is being carried out with rabbit antiserum.

Loss of the group specificity, which was previously observed in members of all three groups on acquiring penicillin resistance, was not seen after induced streptomycin resistance.

Summary. 1. Twenty-three strains of beta hemolytic streptococci of groups A, B, and C were subcultured serially on media containing streptomycin in an effort to induce resistance. The minimum increase in resistance after 40 transfers was 40-fold and the maximum 3000-fold. Most organisms, however, of all 3 groups showed increases of 100- to 400-fold.

2. The streptomycin sensitivity range for recently isolated strains of beta hemolytic streptococci is 10 to 50 $\mu\text{g/ml}$ for 275 strains of group A, 30 to 400 $\mu\text{g/ml}$ for 20 strains of group B, and 10 to 50 $\mu\text{g/ml}$ for 47 strains of group C organisms.

3. Organisms which had acquired streptomycin resistance maintained it throughout subcultivations on blood agar medium.

4. Partial loss of resistance was observed in 5 of 6 strains on serial passages through mice.

5. Mouse virulence was decreased in 8 of 9 resistant organisms studied. Virulence was partially restored by passage through mice.

6. Group specificity was maintained by all resistant organisms.

7. Reduction in streptokinase production occurred in 2 of 23 resistant organisms and streptolysin S in 5 of 5 group A and C strains.

8. Transient changes in the colonial appearance and changes in hemolysis from beta to alpha or gamma types of colonies were demonstrated by all strains when growing on maximal concentrations of streptomycin. These colonies reverted to their original type when subcultured onto blood agar.

We wish to express our appreciation to Dr. C. Phillip Miller for his encouragement and many helpful suggestions in this study.

¹³ Unpublished data.

Serum Concentrations of Penicillin Following Administration of Crystalline Procaine Penicillin G in Aqueous Suspension.

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Previous studies have demonstrated prolonged serum penicillin levels following intramuscular administration of procaine penicillin G in vegetable oil.¹ The disadvantages of penicillin preparations in oil and beeswax consist primarily of an increased incidence of cutaneous sensitivity reactions, the danger of oil embolism, and somewhat decreased ease of administration. The importance of an aqueous penicillin preparation which would insure a prolonged serum penicillin concentration is, therefore, apparent. The purpose of this paper is to present pharmacologic data concerning the use in man of procaine penicillin in aqueous suspension.

Materials and Methods. Antibiotic Agents. Crystalline procaine penicillin G in powdered form was suspended in an aqueous diluent with a dispersing agent.* Later preparations consisted of a mixture of crystalline procaine penicillin G and the dispersing agent, necessitating the addition of either water or physiological saline to form a stable suspension. Dilution to provide a concentration of 300,000 units per cc was made shortly prior to injection. A uniform suspension was obtained by vigorous shaking for several minutes.

Method of Administration. The subjects employed in this study were in the main young adults with no known renal, cardiac, or hepatic damage, many of whom were postpartum females or patients convalescing from some acute illness. Dry, sterile syringes and 18-20-gauge needles were used for the intramuscular administration of either 1 or 2 cc containing

300,000 units of crystalline procaine penicillin G per cc.

Method for Determination of Serum Penicillin Concentrations. Serum penicillin concentrations of these samples were determined by the method of Rammelkamp.²

Results. Administration of 300,000 Units Crystalline Procaine Penicillin G in Aqueous Suspension. Forty-six subjects received a single intramuscular injection of 300,000 units of this suspension with the results shown in Fig. 1 and 2. All subjects within one-half hour showed a level of 0.02 units penicillin per cc or more with 30% attaining levels of at least 0.32 units per cc. The serum penicillin concentrations were uniformly greater 4 hours after administration than at one-half hour with a range of 0.04 to 1.28 units per cc. Twelve hours after administration all subjects had demonstrable levels and 21 (46%) had concentrations of at least 0.08 units per cc serum. Assayable amounts of penicillin were present 24 hours following injection in 36 patients (92%).

Administration of 600,000 Units Crystalline Procaine Penicillin G in Aqueous Suspension. The results of a single intramuscular injection of 600,000 units of this suspension in 32 subjects are recorded in Fig. 3 and 4. Serum penicillin concentrations one-half hour after injection varied between 0.08-1.28 units per cc. Maximum serum levels of 0.04-2.5 units per cc appeared 4 hours after injection. Twelve hours after administration levels varied from 0.04-1.28 units per cc. Twenty-four hours after injection assayable concentrations were present in all subjects with 23 (72%) having a level of 0.08 units per cc. Assayable concentrations were present in

* We are indebted to E. R. Squibb & Sons, New Brunswick, N.J., for supplying the penicillin preparations employed in these studies.

¹ Hewitt, W. L., Whittlesey, P., and Keefer, C. S., *New Eng. J. Med.*, in press.

² Rammelkamp, C. H., *Proc. Soc. Exp. Biol. and Med.*, 1942, **51**, 95.

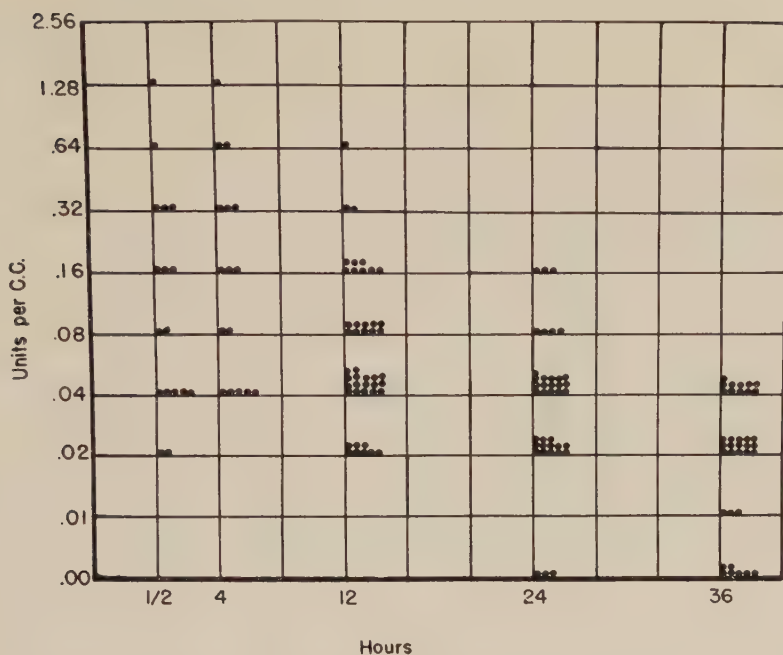


FIG. 1.

Serum penicillin concentrations following administration of 1 cc aqueous suspension containing 300,000 units of crystalline procaine penicillin G.

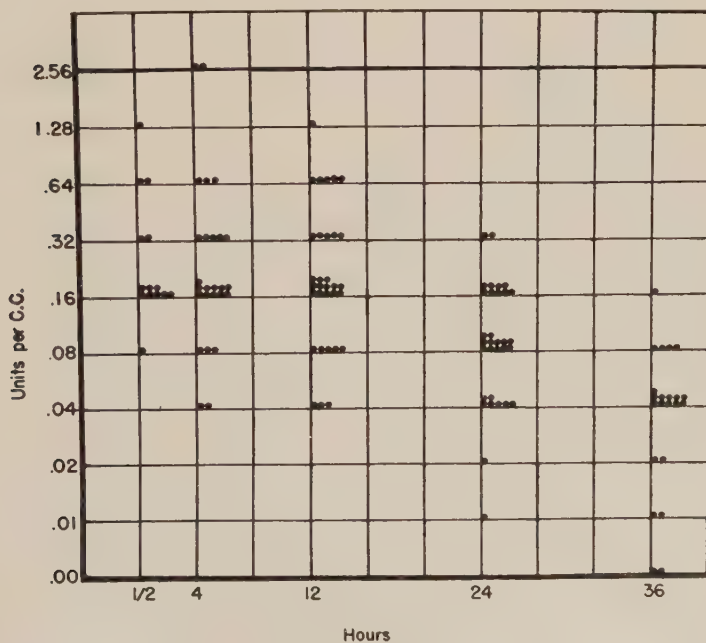


FIG. 2.

Serum penicillin concentrations following administration of 1 cc aqueous suspension containing 600,000 units of crystalline procaine penicillin G.

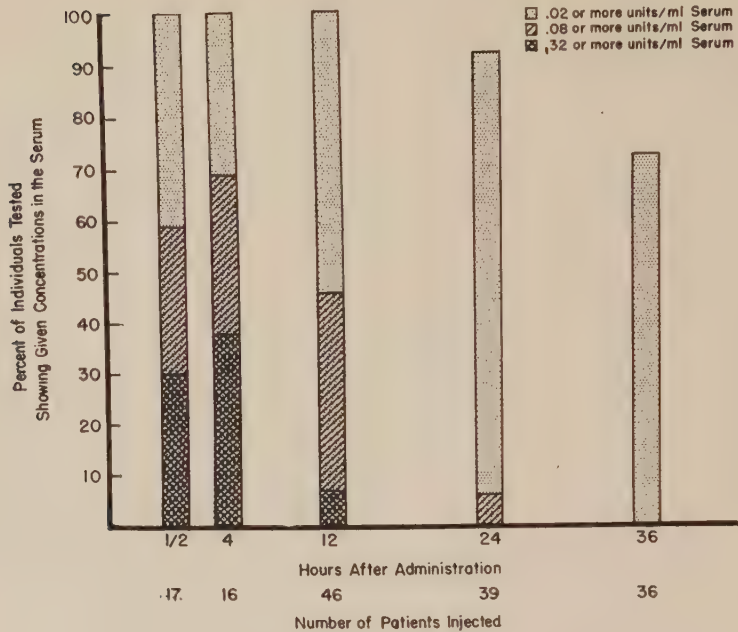


FIG. 3.

Incidence of given serum penicillin concentrations following administration of 1 cc aqueous suspension containing 300,000 units of crystalline procaine penicillin G.

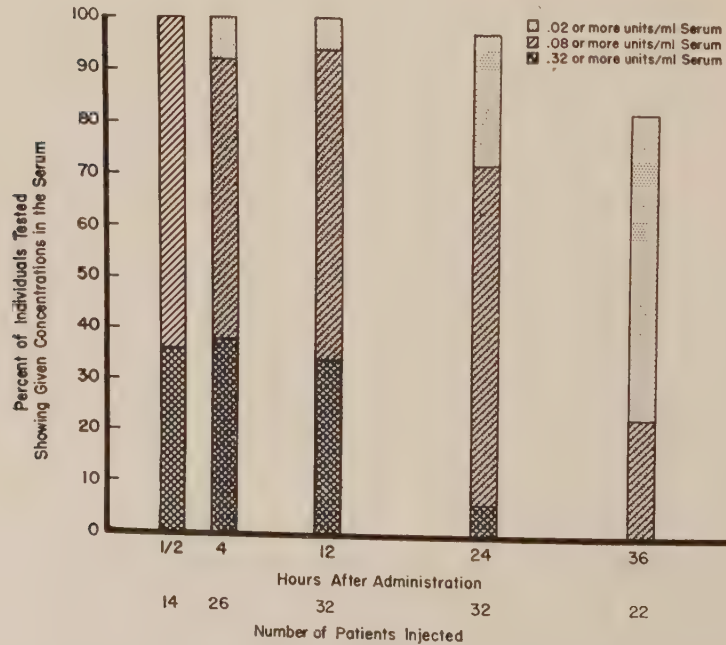


FIG. 4.

Incidence of given serum penicillin concentrations following administration of 1 cc aqueous suspension containing 600,000 units of crystalline procaine penicillin G.

94% of subjects 36 hours after injection.

Comment. Ease of administration characterized the injections of procaine penicillin in aqueous suspension. Clogging of needles occurred occasionally due to aggregation of procaine penicillin crystals. The frequency of this may be minimized by thorough shaking of the preparation prior to withdrawal into the syringe, immediate injection, and use of 18 or 19 gauge needles for administration. Pain, inflammatory reaction or subcutaneous nodules were not observed at the site of injections. No systemic reactions occurred.

The serum penicillin concentrations following similar doses of vegetable oil suspension and aqueous suspension of procaine penicillin G manifested the same general pattern differing from peanut oil-beeswax preparations by the absence of a high initial peak shortly after injection and the presence of a more uniform serum level during the 24 hours after administration.¹ However, it was noted that with 300,000 units a slightly higher percentage of patients maintained a level of 0.02 units per cc or more but a lower percentage maintained 0.08 units per cc with the aqueous suspension. In using 600,000 units the aque-

ous suspension group showed a lower percentage throughout of comparable serum levels.

Summary. 1. Administration of 300,000 units of crystalline procaine penicillin G in aqueous suspension produced demonstrable serum penicillin concentrations at 12 hours after administration in all subjects and at 24 hours after injection in 92% of subjects studied.

2. Administration of 600,000 units of this material produced demonstrable serum penicillin concentrations 24 hours after injection in all subjects.

3. Similar patterns of serum penicillin concentrations were obtained for both the oil and the aqueous suspensions of procaine penicillin G although slightly lower values were generally observed with the aqueous suspension.

4. Ease of administration and absence of untoward local or systemic reaction were uniformly observed with the administration of procaine penicillin G in aqueous suspension.

We are indebted to Miss Doris McCarthy for technical assistance.

16587 P

The Effect of Streptomycin on Well-Established Experimental Tuberculosis of Mice.*

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Previous studies have shown streptomycin to be effective for the suppression of experimental tuberculosis in mice when treatment was started at the time the animals were infected.^{1,2,3} Although well-established tuberculosis of guinea pigs is known^{4,5,6,7} to be favorably affected by streptomycin therapy,

there is no information available as to the degree of effectiveness of this agent on well-established tuberculous infections of mice.

¹ Young, G. P., *Quart. Bull., North. Univ. Med. School*, 1945, **19**, 207.

² Young, G. P., and McCarter, J. C., *Am. Rev. Tuberc.*, 1945, **52**, 432.

³ Young, G. P., and Williston, E. H., *Proc. Soc. Exp. Biol. and Med.*, 1946, **63**, 131.

⁴ Feldman, W. H., Hinshaw, C. T., and Mann, F. C., *Am. Rev. Tuberc.*, 1945, **52**, 269.

* This investigation was supported (in part) by a research grant from the Division of Research Grants and Fellowships of the National Institute of Health, U. S. Public Health Service.

TABLE I.
Amount of Pulmonary Tuberculosis in Mice Sacrificed at 7, 14, and 21 Days After Infection with *M. tuberculosis*.

No. of mice	Time sacrificed in days	Avg amt of gross pulmonary tuberculosis	Avg amt of microscopic pulmonary tuberculosis	Type of pulmonary lesions
15	7	0.8+	1.0+	P
15	14	2.8+	2.0+	P
15	21	3.8+	3.5+	NE and P

P—Proliferative lesions.

NE—Necrotic exudative lesions.

1+—0-10% of lung substance involved.

2+—11-25% of lung substance involved.

3+—26-50% of lung substance involved.

4+—Over 50% of lung substance involved.

Methods. Two separate experiments were conducted using white mice infected intravenously with 0.1 mg of the virulent H37Rv strain of *M. tuberculosis* var. *hominis*.² In the first experiment groups of mice were treated with 3000 μ g of streptomycin given daily approximately 6 hours apart in 4 subcutaneous doses of 750 μ g each. Treatment of the first group was started the day the mice were infected, while treatment of 3 other groups was started at 7, 14 and 21 days after infection. A fifth group received no streptomycin and served as a control on the other groups. A second similar experiment was performed, with the exception that the streptomycin was administered in 2 daily subcutaneous doses of 1500 μ g approximately 8 hours apart. This alteration in technic was employed since other experiments in the meantime had shown that the degree of suppression of the tuberculous process, employing 3000.0 μ g streptomycin daily, was essentially the same regardless of whether the streptomycin was given in 2 or 4 daily injections.

All animals, except the controls, were treated with streptomycin for 28 days, following which, therapy was discontinued and the mice observed until death supervened. At autopsy the viscera of all mice were fixed in 3.7%

formaldehyde, and the amount of gross and microscopic tuberculosis determined and recorded as previously described.^{8,9}

Three other groups of untreated mice were sacrificed at 7, 14 and 21 days respectively after infection with tubercle bacilli. The pathological findings in these animals served as an indication of the amount and type of pulmonary tuberculosis present at the time treatment of the other groups with streptomycin was started.

Results. Table I shows the amount of tuberculosis present in the animals sacrificed at 7, 14 and 21 days. The findings in these animals were similar to those previously reported,² and indicated the presence of well-established tuberculosis, even as early as 7 days after infection.

Table II indicates the results obtained in the first experiment with those mice in which treatment was started at 0, 7, 14, and 21 days after inoculation, and with the untreated controls.

Table III shows similar data for the mice used in the second experiment. The only animals included in the tables were those from which complete and suitable tissue specimens were available for pathological examination.

The numbers of animals in the groups in the first experiment (Table II) are rather small, but there is a significant difference between the average time of survival of the

⁵ Smith, M. I., and McCloskey, W. T., *U. S. Pub. Health Report*, 1945, **60**, 1129.

⁶ Callomon, F. T., Kolmer, J. A., Rule, A. M., and Paul, A. J., *Proc. Soc. Exp. Biol. and Med.*, 1946, **63**, 237.

⁷ Steenken, W., Jr., and Wolinsky, E., *Am. Rev. Tuberc.*, 1947, **56**, 227.

⁸ Raleigh, G. W., and Youmans, G. P., *J. Inf. Dis.*, 1948, **82**, 205.

⁹ Youmans, G. P., and Raleigh, G. W., *J. Inf. Dis.*, 1948, **82**, 221.

TABLE II.
Effect of Streptomycin on Well-Established Experimental Tuberculosis in Mice.

No. of mice	Days after infection treatment started	Streptomycin daily (μ g)	Avg survival time (days)	Standard deviation	Pulmonary tuberculosis		
					Gross	Microscopic	Type lesion
7	0	3000.0	108.1	± 36.1	2.4+	3.0+	P
11	7	3000.0	118.5	± 27.2	3.9+	3.7+	P
8	14	3000.0	134.6	± 30.6	3.6+	3.5+	P
				P = 0.2			
11	21	3000.0	109.0	± 44.3	3.1+	3.5+	P
				P = <0.01			
13	0		38.0	± 27.4	3.7+	4.0+	NE

TABLE III.
Effect of Streptomycin on Well-Established Experimental Tuberculosis in Mice.

No. of mice	Days after infection treatment started	Streptomycin daily (μ g)	Avg survival time (days)	Standard deviation	Pulmonary tuberculosis		
					Gross	Microscopic	Type lesion
16	0	3000.0	115.3	± 31.6	3.8+	4.0+	P
18	7	3000.0	105.1	± 29.6	3.5+	3.1+	P
17	14	3000.0	112.6	± 15.3	3.6+	4.0+	P
15	21	3000.0	94.6	± 58.3	3.8+	4.0+	P
				P = <0.01			
29	0		20.6	± 2.8	3.8+	4.0+	NE

treated animals as compared with the controls.

The numbers of animals employed in the second experiment were larger, and the results were essentially similar to those of the first experiment, even though, as indicated by the average survival time of the controls, the disease was somewhat more severe in the second experiment. Statistical analysis of the data in Table III indicates a significant difference between the average survival time of the control and streptomycin treated animals, but not between the groups of treated animals. The somewhat lower average survival time of the groups of mice in which treatment was delayed 21 days reflects the loss of several animals within 2 days following initiation of therapy. These animals were apparently moribund and derived no benefit from therapy—had they been excluded from the data

the average survival time would have been even more similar to those of the 7 and 14 day groups.

The physical appearance of the mice confirmed the statistical data. Most of the mice in the 21-day group had lost from 3 to 6 g in weight, had ruffled fur and looked emaciated and listless. With the exception of the mice that died in 2 days, all the others began to improve in appearance and to gain weight 3 to 5 days following the institution of streptomycin therapy. By the end of 28 days' treatment normal weight had been restored.

All the mice eventually died and the extent of the pulmonary disease did not differ significantly from the control animals. All the treated subjects however exhibited proliferative lesions, whereas the lesions of the

controls were predominantly necrotic exudative. The lesions in the treated animals were extensive, occupied almost all alveoli, but the numbers of tubercle bacilli were considerably reduced as compared with the controls. The livers and spleens of such animals were extremely congested and this, in combination with the great reduction in space for gas exchange in the lungs, suggests that death may have resulted primarily from anoxia and right heart failure rather than from the toxic effects of the tuberculous infection.

These findings indicate that mice may be benefited even late in the course of tuberculosis induced by intravenous inoculation. All

groups, whether treated from the day of inoculation or from the 7th, 14th, or 21st days had the same amount of disease on death, and since the average survival times were not significantly different, it would seem that the subjects with most severe disease at the time treatment was started must have obtained relatively greater benefit from streptomycin. The explanation for this phenomenon, however, is not readily apparent.

Conclusion. Streptomycin to the amount of 3000 μ g per day had a favorable effect upon the course of experimental tuberculosis in mice when treatment was initiated 7, 14 and 21 days following infection.

16588

A New Chromosome Stain and Its Relationship to Atypical Cell Proliferation.

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The metabolic transformations of paraphenylenediamine and certain azo-dyes into quinone diimine (Mayer¹) are accompanied by staining effects on epidermal and other cells. This would indicate that quinone diimine possesses a strong affinity for certain nuclear material to which it becomes attached. Since it seemed probable that the cell proliferations with precancerous and cancerous degeneration produced by aromatic amines are caused by the affinity of their oxidation products for certain vital components of the cell nuclei, we have investigated that part of the nucleus with which quinone diimine combines. We have therefore attempted to stain chromosomes with the oxidation products of paraphenylenediamine, and several of its derivatives.

Certain substitution products of paraphenylenediamine, such as dimethyl- and tetramethyl paraphenylenediamine and also meta-

phenylenediamine, have been used as microscopic or vital stains by Wurster,^{2,3} Unna⁴ and Joseph and Wurster.⁵ In combination with β -naphthol, dimethyl paraphenylenediamine forms blue indophenol in the presence of oxidative cell enzymes (Ehrlich⁶), a reaction known as the "Nadi reaction". No reference in the literature has been found on similar uses of paraphenylenediamine itself.

Methods. Source of chromosomes. Salivary glands of *Drosophila melanogaster* and *Drosophila robusta* larvae* maintained on molasses agar were prepared in the usual

² Wurster, C., *Ber. Dtsch. Chem. Ges.*, 1886, **19**, 3195.

³ Wurster, C., *Ber. Dtsch. Chem. Ges.*, 1887, **20**, 256.

⁴ Unna, P. G., *Monatsh. Prakt. Dermat.*, 1887, **6**, 62.

⁵ Joseph, M., and Wurster, C., *Monatsh. Prakt. Dermat.*, 1887, **6**, 243.

⁶ Ehrlich, P., *Das Sauerstoffbedürfnis des Organismus*, Berlin, 1885.

¹ Mayer, R. L., *J. Invest. Dermatol.*, 1948, **10**, 389.

way (Darlington and LaCour⁷). The isolated glands were suspended on a microscope slide in a drop of 50% acetic acid.

Staining solutions prepared from paraphenylenediamines. 0.2 g of paraphenylenediamine or its derivatives, namely *s*-dimethyl paraphenylenediamine dioxalate and tetramethyl paraphenylenediamine hydrochloride were dissolved in 20 ml of hot 20% acetic acid. 0.1 ml of 30% hydrogen peroxide was added and the mixture cooled as soon as a light-brown color had developed. The oxidation continues slowly and after approximately 30 minutes the stain is ready to use. The solutions of paraphenylenediamine are then dark-brown and those of the 2 derivatives red and purple respectively.

Another staining solution prepared from pure quinone compounds is described under staining process, part b.

Staining process. a). *Stain prepared from aqueous solutions of paraphenylenediamine.* This stain is very sensitive to certain metals; traces of iron, dissolved from dissecting needles in contact with acetic acid, suffice to rapidly produce dark-purple or brown colorations and precipitations. Such an iron-containing stain provides excellent specimens, but has the disadvantage that the protoplasm and connective tissue stain more darkly than in absence of iron. It is therefore preferable to avoid contamination with iron by removing the acetic acid solution, in which the salivary glands are suspended and which had been in contact with the dissecting needles with filter paper before adding the stain. The slides are stored for one hour in Petri dishes over moist filter paper, then covered with a cover glass and squash preparations made in the usual way. If the coloration is too light, another drop of the staining solution may be placed under the cover glass for an additional 30 minutes.

Particularly good preparations were ob-

tained when the glands were pulped in the acetic acid solution before staining.

b). *Stain prepared with pure quinone diimine.* Since the oxidation of paraphenylenediamine with hydrogen peroxide in water furnishes a large number of darkly colored intermediate and polymerization products, pure quinone diimine was prepared according to Willstaedter and Pfannenstiel.⁸ Freshly precipitated silver oxide is thoroughly washed with water, followed by anhydrous acetone and ether to remove all traces of salts and water and then suspended in absolute ether. 0.2 g of paraphenylenediamine base are then dissolved in 100 ml of absolute ether, 1.0 g of the water-free silver oxide added and the mixture shaken for 2 hours. After filtration the ether is removed on a water bath. The yellow to reddish crystals remaining are very unstable, and the quinone diimine crystals must be freshly prepared for each staining series. The following procedure gives the best results: Salivary glands are suspended in 50% acetic acid. Ten mg of the quinone diimine crystals are dissolved in 1 cc of 70% acetic acid; the solution turns dark-blue with formation of a deeply-colored precipitate, which dissolves in an excess of acetic acid. A weakly-colored solution results which may be added immediately to the chromosomes placed on the slide; the development of the stain in this case requires 10 to 20 minutes.

Instead of isolating the quinone diimine from the solvent, the ether solution of the quinone diimine may be used directly for staining. Salivary glands are suspended in 50% acetic acid, crushed preparations made and the material re-suspended in a drop of 70% acetic acid. A drop of the ether solution containing the quinone diimine as prepared according to Willstaedter⁸ is then added. Again, immediately upon contact with the acetic acid solution the quinone diimine is transformed into a deep-blue compound which re-dissolves immediately in excess of acid to yield a light-brownish color. About one-half hour later the chromosomes are very dark, purple-brownish, whereas the protoplasm in

* We wish to thank Dr. Max Levitan, Columbia University, for the strain of *Drosophila robusta* as well as for much valuable technical information.

⁷ Darlington, C. D., and La Cour, L. F., *The Handling of Chromosomes*, 2nd Edition, London, 1947.

⁸ Willstaedter, R., and Pfannenstiel, A., *Ber. Dtsch. Chem. Ges.*, 1904, **37**, 4605.

most instances remains almost uncolored. This procedure has not only given the best results, but has also prevented the formation of precipitates on the slides.

From the practical standpoint, however, the staining procedure in which aqueous solutions of paraphenylenediamine are oxidized with hydrogen peroxide and the resulting solution used as stain, is the simplest and provides specimens of sufficient contrast for most purposes.

c). *Stains prepared from s-dimethyl- and tetramethyl paraphenylenediamine, quinone chlorimine and quinone dichlorimine.* The dimethyl and tetramethyl derivatives of paraphenylenediamine oxidized in water with H_2O_2 as described above and applied to the salivary glands under the same conditions as oxidized paraphenylenediamine or quinone diimine, failed to stain the nuclei and chromosomes. Similar negative results were regularly obtained using pure quinone chlorimine and pure quinone dichlorimine. In either case the protoplasm became distinctly colored and showed the sites of the nuclei as uncolored areas. The substituted quinone diimines have a much lower basicity than unsubstituted quinone diimine, which apparently prevents their combination with nuclear material and favors the combination with protoplasm protein.

d). *The staining of chromosomes at pH 6.5.* It is possible to stain chromosomes as well as nucleoli with 1 to 10% paraphenylenediamine diacetate dissolved in pH 6.5 acetate buffer. The purple stain, which develops upon exposure to air within several hours, is more diffuse than when made at lower pH values.

Microscopic aspect. In properly stained preparations the nuclei appear as dark-brown spots on a yellowish-brown background. The chromosomes disclose a typical succession of broader or narrower colored and uncolored bands and granulations, the color of these bands or granules ranging from brown to deep-black, often with a definite purple component (Fig. 1 and 2).

Contrary to the chromosome specimens stained with aceto-carmin or aceto-orcein

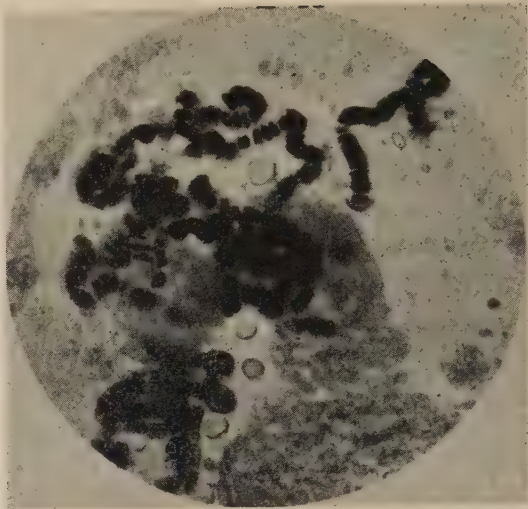


FIG. 1.
Chromosomes stained with oxidized paraphenylenediamine.

which fade within a short time, the color of specimens stained with quinone diimine is stable for many weeks. Often the color further deepens on standing.

Discussion. Specimens of chromosomes stained with quinone diimine very closely resemble, in their general aspect, chromosomes stained with the usual chromosome dyes. Similarly, a comparison of the distribution of the bands in specimens stained by the various staining methods strongly suggests that the quinone diimine stain affects the same areas, and, consequently, the same components of the chromosomes which are stained with aceto-carmin, aceto-orcein, or the Feulgen technic.

Considering the chemical reactions characteristic for quinone diimine and other compounds of quinone structure, we believe that the quinone diimine chromosome stain is based upon the formation on an anilido quinone-like fixation of quinone diimine to the nucleoproteids of the chromosomes, *i.e.* the genes.

Previous experiments¹ have suggested that a combination of compounds of quinone structure, especially quinone diimine, with proteins and nucleoproteids takes place within the living body when certain aromatic amines are introduced and there produce pathological reactions.



FIG. 2.
Chromosomes stained with quinone diimine.

It is known from clinical experiences and animal investigations that aromatic amines such as paraphenylenediamine, naphthylamine and certain azo-dyes containing aromatic amino groups such as butter yellow and scarlet red produce allergic sensitizations—asthma and dermatitis—and atypical epithelial proliferations (Fischer,^{9,10} Sachs,¹¹ Martinotti¹²), as well as cancer (Yamagiwa and Ohno,¹³ Hueper,^{14,15,16} Sasaki and Yoshida,¹⁷ Yo-

shida^{18,19} Miller and co-workers²⁰ and Harris and co-workers²¹). There are many indications that these amines are only the precursors of the directly active principle which is formed within the body after a series of metabolic transformations.

Azo-dyes, serving as hydrogen acceptors, are reduced to amines and diamines, especially paraphenylenediamine, which are then oxidized into quinone diimine and related compounds of quinone structure (Mayer²²⁻²⁵). Aromatic amines or paraphenylenediamine

⁹ Fischer, B., *Munch. Med. Wschr.*, 1906, **53**, 2041.

¹⁰ Fischer, B., *Frankf. Z. Path.*, 1922, **27**, 98.

¹¹ Sachs, O., *Arch. f. Dermat.*, 1913, **116**, 559.

¹² Martinotti, *Berl. Klin. Wschr.*, 1914, 1441.

¹³ Yamagiwa, K., and Ohno, S., *Jap. Z. f. Krebsf.*, 1918, **12**, 3.

¹⁴ Hueper, W. C., Willy, F. H., and Wolfe, H. D., *J. Indust. Hyg. and Toxicol.*, 1938, **20**, 26 and 85.

¹⁵ Hueper, W. C., *Arch. Path.*, 1938, **25**, 856.

¹⁶ Hueper, W. C., Briggs, F. A., and Wolfe, H. D., *J. Indust. Hyg. and Toxicol.*, 1938, **20**, 85.

¹⁷ Sasaki, T., and Yoshida, T., *Virchows Arch. Path. Anat.*, 1935, **295**, 175.

¹⁸ Yoshida, T., *Transact. Japan. Path. Soc.*, 1933, **23**, 636.

¹⁹ Yoshida, T., *Transact. Japan. Path. Soc.*, 1934, **24**, 523.

²⁰ Miller, J. A., Kline, B. E., Ruseh, H. P., and Baumann, C. A., *Cancer Research*, 1944, **4**, 153.

²¹ Harris, M., Krah, M. E., and Clowes, G. H. A., *Cancer Research*, 1947, **7**, 162.

²² Mayer, R. L., *Arch. f. Dermatol.*, 1928, **156**, 331.

²³ Mayer, R. L., *Arch. f. Dermatol.*, 1929, **158**, 266.

are directly oxidized. The first phase of these metabolic transformations, namely the reduction of azo-dyes to paraphenylenediamine, has been studied by Nitti and co-workers²⁶ and by Stevenson, Dobriner and Rhoads.²⁷ An interesting example of a biological oxidation of an aromatic amine associated with the production of cancer has been described by Haddow.²⁸

Contrary to the slowly reacting amines and azo-dyes, most quinone derivatives are biologically very active, being strong oxidizing agents. However, the property in which we are especially interested is their ability to combine with numerous aromatic and aliphatic compounds to form anilido quinones or anilido quinone imines (Fischer,²⁹ Posner,³⁰ Martynoff and Tsatsas,³¹ Suida and Suida³²). Among the body constituents with which quinone diimine readily combines are various amino acids, especially those which contain -S-S or SH groups, proteins and nucleoproteids.

We have explained the high allergic activity of paraphenylenediamine as the result of the formation of anilido quinone-like combination with body proteins, and the production of atypical cell proliferation with the

formation of similar combinations with nucleoproteids (Mayer^{1,22}).

As the present study on chromosome stains suggests, an anilido quinone-like fixation of metabolically formed quinone diimine to chromosomes or genes may readily take place when sufficient amounts of aromatic amines or azodyes are introduced into the body. This combination with the specific material of the chromosomes is very strong, the chromosome stain resisting a great number of chemical influences; it is strong enough to account for disturbances in the physiological behavior of the chromosomes and genes.

Further experiments are necessary to prove that it really is such a blockade of the chromosomes and the combination of genes with quinone derivatives as a consequence of contact with aromatic amines which leads to atypical proliferations of the affected cells and, in certain cases, even to mutations and malignant degeneration.

Besides its general interest with respect to the problem of atypical cell proliferations and cancer, the quinone diimine stain possesses certain technical advantages over the known chromosome stains. In many instances the chromosomes made visible by our procedure are more distinct than with other stains; moreover, in varying the time of exposure to the stain it is possible to obtain wide variations in the intensity of coloration, thus enhancing or suppressing certain features.

Conclusion. A new chromosome stain is described which is based upon the affinity of nucleoproteids for certain quinone derivatives. The relationship between this stain and the production of atypical epithelial proliferations and cancer produced by aromatic amines is discussed.

Acknowledgment is made to Miss Jeanne Long, Parasitology Laboratory, for maintaining the strains of *Drosophila*.

²⁴ Mayer, R. L., *Arch. f. Dermatol.*, 1931, **163**, 223.

²⁵ Mayer, R. L., *Arch. f. Gewerbepathol.*, 1930, **1**, 436.

²⁶ Nitti, F., Bovet, D., and Depierre, F., *C. R. Soc. Biol.*, 1937, **124**, 1164.

²⁷ Stevenson, E. S., Dobriner, K., and Rhoads, C. P., *Cancer Research*, 1942, **2**, 160.

²⁸ Haddow, A., *Brit. Med. Bull.*, 1947, **4**, 1.

²⁹ Fischer, E., and Schrader, H., *Ber. Dtsch. Chem. Ges.*, 1910, **43**, 525.

³⁰ Posner, T., *Liebigs Ann.*, 1904, **336**, 108.

³¹ Martynoff, M., and Tsatsas, G., *Bull. Soc. Chim. France*, 1947, 52.

³² Suida, H., and Suida, W., *Liebigs Ann.*, 1918, **416**, 113.

Nutritive Value of Casein as Influenced by Alcohol Extraction.*

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Since casein is an important constituent of most purified rations, methods of preparing and purifying this protein are of importance to all nutritionists. Many workers have used alcohol extraction and there is definite evidence that traces of vitamins remaining in crude casein are removed by the procedure. Hartman and Cary^{1,2,3,4} found that rats grow at a much slower rate when fed casein thoroughly extracted with 95% alcohol than when fed the untreated casein. They believe that an unknown factor necessary for the normal growth of the rat is removed and they termed the substance factor X, or a factor of palatability. The reduced rate of growth of rats fed the alcohol extracted casein was accompanied by a decreased food consumption. Normal growth was secured by supplementing the alcohol extracted casein ration with certain liver concentrates.

The following study was undertaken in order to determine if similar results could be obtained under our experimental conditions and if the alcohol treatment could have any effect on the release of the amino acids in the treated casein.

Experimental. Pregnant rats of the Sprague-Dawley strain, were placed at par-

turition on a diet consisting of sucrose or dextrin 55%, alcohol extracted casein 20%, cotton seed oil containing the fat soluble vitamins (Table I) 10%, brewers yeast 10%, and salts IV (Hegsted *et al.*⁵) 5%. When the young from these females were 21 days of age they were placed in individual cages with raised screen floors. Care was taken to ensure equal distribution of the young on the basis of weight, sex, and parentage. Experimental diets and water were fed *ad libitum* and individual body weights were recorded weekly over a 5 week period. Composition of the rations and the total weight gains are given in Table I.

The extracted casein was prepared by boiling 2.5 kg of crude casein with 4 liters of 95% ethanol for 8 hours with continuous stirring. This procedure was repeated 10 successive times. It is evident from Table I, Experiments 1 and 2, that a definite decrease in rate of growth occurred in all the male rats receiving extracted casein as compared to those receiving the ration containing crude casein. The female rats showed a much smaller difference.

For the following experiments the extraction of the casein with ethanol was increased to 10-24 hour periods, and dextrin was used as the carbohydrate. With this more thoroughly extracted casein greater differences were observed with both male and female rats. When the protein was increased to 40% of the ration, the difference was somewhat less but could be attributed to the reduced rate of growth of the rats fed the high level of crude casein. Likewise, when the yeast supplement was replaced by synthetic vitamins, the rate of growth over the 5 week experimental period was decreased significantly when

* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. This work was supported in part by grants from the Evaporated Milk Association, Chicago, and the National Dairy Council, Chicago, in behalf of the American Dairy Association.

[†] Government of India Research Fellow.

¹ Cary, C. A., Hartman, A. M., Dryden, L. P., and Likely, G. D., *Fed. Proc.*, 1946, **5**, 128.

² Cary, C. A., and Hartman, A. M., *Fed. Proc.*, 1946, **5**, 137.

³ Cary, C. A., and Hartman, A. M., *J. Am. Diet. Assn.*, 1946, **22**, 1016.

⁴ Cary, C. A., and Hartman, A. M., *U. S. Dept. Agr. Pub.*, Sept., 1947.

⁵ Hegsted, D. M., Mills, R. C., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, 1940, **138**, 459.

TABLE I.
Composition of Rations* and Growth Rate of Rats.

Exp. No.	Group	Crude casein	Extracted casein	Sucrose	Dextrose	Dextrin	Brewers yeast	Vit.†	Supplement	5-wk wt gain‡	
										Male g	Female g
1	1		20		55		10			150	114
	2	20			55		10			175	118
	3		25	60				x		158	117
	4	25		60				x		176	123
2	1		20		55		10			159	115
	2	20			55		10			—	124
	3		25	60				x		114	80
	4	25		60				x		148	117
3	1		20			55	10			85	85
	2	20				55	10			166	122
	3		40			35	10			103	64
	4	40				35	10			149	104
	5		40	45				x		107	74
	6	40		45				x		122	92
4	1		20			55	10			121	93
	2	20				55	10			123	108
	3		20			55	10	x	Crude concentrate	111	78
5	1		20			55	10			135	118
	2	20				55	10			188	131
	3		20			55	10			127	108
6	1		20			55	10			124	81
	2	20				55	10		Purified concentrate	185	106
	3		20			55	10			148	114
7	1		20			65		x		154	120
	2		20			65		x	Liver powder‡	197	132
	3	20				65		x		188	140
	4	20				65		x		141	109
8	1		20			65		x		177	136
	2		20			65		x	Fresh liver	190	138
	3	20				65		x			

* All rations contained salts IV 5%, and vitamin supplemented cottonseed oil 10%. 500 g of cottonseed oil was supplemented with 7.52 mg 2-Me-1,4-naphthoquinone, 0.502 mg calciferol, 20.0 mg β -carotene, and 1.08 mg α -tocopherol.

† Each 100 g of ration contained 0.50 mg thiamin, 0.60 mg pyridoxine, 0.70 mg riboflavin, 3.00 mg calcium pantothenate, 150 mg choline hydrochloride, 0.60 mg nicotinic acid, 0.025 mg biotin, 0.20 mg folic acid, 15.00 mg *p*-aminobenzoic acid and 50.0 mg inositol.

‡ Each figure represents the average weight of 3 animals.

§ Wilson solubilized liver powder (Fraction "I,").

TABLE II.
Amino Acid Content of Raw and Alcohol Extracted Casein After Hydrolysis with Acid or Enzymes.

	Acid hydrolysis*		Enzyme hydrolysis†	
	Raw	Alcohol extracted	Raw	Alcohol extracted
Arginine	3.54	3.27	4.48	4.11
Aspartic acid	6.50	6.82	0.3	0.5
Cystine	0.20	0.17	0.08	0.06
Glutamic acid	20.1	20.9	11.0	9.75
Histidine	2.46	2.55	1.14	1.04
Isoleucine	5.05	5.26	5.93	4.09
Leucine	9.0	9.15	8.82	8.77
Lysine	6.45	6.35	4.69	4.7
Phenylalanine	4.77	5.1	4.43	4.27
Proline	10.1	10.3	3.70	3.60
Threonine	3.87	4.25	3.48	3.36
Tryptophan	1.05	1.16	1.13	1.08
Tyrosine	4.1	4.25	4.08	4.6
Valine	6.45	6.92	5.86	5.54
α -Amino nitrogen‡	100	100	53	55

* Casein was autoclaved for 10 hours with 25 vol. of 6 N HCl at 15 lbs pressure, except in the case of tryptophan, where 20 vol. of 5 N NaOH was used and autoclaved for 5 hours at 15 lbs pressure.

† 1 g of casein was digested with 100 mg pancreatin (Merek + 20 mg hog intestinal mucosa (Wilson), with shaking at 37°C for 3 days at pH 8-6, after making the volume to 100 ml.

‡ Per cent liberation of α -amino nitrogen (Van Slyke) based upon complete liberation of amino groups after acid hydrolysis.

the extracted casein was used.

Rats ingesting the more completely extracted casein developed a characteristic hair loss on the dorsal, anterior and posterior areas of the body. This hair loss gradually enveloped the entire dorsal region of the animal.

The alcohol filtrates were reduced to a volume of 200 cc and used as a crude concentrate. A more purified concentrate was prepared by allowing the crude filtrate to stand in the cold (-4° C) for 24 hours, filtering and drying the precipitates at 65° C.

The addition of either the crude or the purified alcohol concentrate from casein at a level equivalent to 20% casein (Experiments 4 and 6) failed to enhance growth but did prevent hair loss. In all cases, the use of crude unextracted casein increased the rate of growth. The addition of fresh liver at the rate of one gram per rat per day brought the rate of growth of the rats receiving extracted casein to normal and also prevented hair loss. Solubilized liver powder at a level of 2% in the ration did not affect the growth rate or hair loss.

In order to determine if the reduced nutritive value of the casein was due to the

loss of or change in any of the constituent amino acids, the raw and extracted casein were analyzed for 14 different amino acids using the method of Henderson and Snell.⁶ In one series the determinations were made after acid hydrolysis and in the other series enzymatic digestion was used in order to detect any possible difference in the rate of release. The results are given in Table II and it is evident that the values for the 2 caseins are almost identical. Methionine values are not recorded because the standard curve was not acceptable. However, there was no difference in the response of the test organism to the hydrolysates prepared from the two caseins. The values for aspartic acid and glutamic acid in the enzyme hydrolysates may not be true values since no attempt was made to determine asparagine and glutamine. Since not all the amino acids especially serine were studied it is still possible that changes occur in certain amino acids which are responsible for the observed difference in nutritive value. It is also possible that the *in vitro* methods used do not give a true indication of the *in vivo*

⁶ Henderson, L. M., and Snell, E. E., *J. Biol. Chem.*, 1948, **172**, 15.

release of amino acids from the two caseins.

If the greater nutritive value of crude casein is due to an impurity, this growth promoting factor must be fairly heat stable since it is present in crude casein which is heated during its preparation. However, after extraction with alcohol and concentration of the extracts, the activity cannot be recovered. The fact that the extract prevents hair loss indicates that either a partial loss of the factor occurred during concentration or that two factors are involved—one with prevention of hair loss and one with growth.

It is also important to point out that the alcohol treatment used may produce toxic substances which appear in both the extracted casein and the alcohol extracts. The fact that similar results were obtained with sucrose, dextrose and dextrin and the fact that the addition of the concentrates gave further weight retardation, give added weight to this possibility. Food consumption records also showed a decreased food consumption associated with the reduced growth. If the effect

is due to the presence of a toxic factor, the addition of fresh liver overcomes this action either by counteracting the toxicity or by supplying factors which cannot be produced by intestinal flora in the presence of the toxic material.

Summary. The nutritive quality of crude casein is reduced by continuous extraction with hot 95% ethanol. These results confirm the observations of Hartman and Cary. Rats fed rations containing such extracted casein grow at a reduced rate and develop a characteristic hair loss. The alcohol extracts obtained from the extraction of crude casein do not increase the rate of growth of rats ingesting extracted casein but do prevent the characteristic hair loss. The growth rate of rats ingesting alcohol extracted casein can be increased to normal by feeding fresh liver. The analysis of the extracted casein and the crude casein for 14 amino acids following enzymatic or acid hydrolysis failed to show any appreciable differences.

16590

Some Histological Manifestations of a Tryptophan Deficiency in the Chick.

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Although many investigations have been conducted on the effect of amino acid deficiencies on the growth rate and on the amino acid composition of tissues and excreta, few studies have been conducted on the histological changes in the various tissues attributable to the ingestion of amino acid deficient diets by the animal. Particular attention however, has been given to changes in blood composition, primarily in the level of hemoglobin and plasma proteins.¹ Other studies

have revealed corneal vascularization resulting from various amino acid deficiencies^{2,3} and atrophy of the thymus, adrenal cortex, liver and epithelial cells of the seminiferous tubules when leucine deficient diets were fed to rats.⁴ Similar studies have been conducted

¹ Cannon, P. R., *Advances in Protein Chemistry*, 1945, **2**, 135.

² Sydenstricker, V. P., Schmidt, H. L., and Hall, W. K., *Proc. Soc. Exp. Biol. and Med.*, 1947, **64**, 59.

³ Sydenstricker, V. P., Hall, W. K., Bowles, L. L., and Schmidt, H. L., *J. Nutrition*, 1947, **34**, 481.

⁴ Maun, M. E., Cahill, W. M., and Davis, R. M., *Arch. Path.*, 1945, **40**, 173.

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with rats fed histidine or phenylalanine deficient diets.^{5,6}

In the course of studies on the metabolism and dietary requirement of tryptophan by the chick,⁷⁻⁹ it was of interest to determine the histology of various tissues from chicks fed diets deficient in tryptophan, adequate in tryptophan, or adequate in tryptophan with the food intake restricted to that of the deficient group.

Experimental and Results. The purified basal diet used has been described previously.⁷ The major protein components were supplied by oxidized casein,¹⁰ gelatin, fish solubles, cystine and methionine. The remainder of the ration consisted of starch, corn oil, mineral mixture and adequate quantities of all vitamins. The control chicks used for histological study received the basal diet plus *L*-tryptophan added in excess of the minimum requirement.⁸ The food allocated to the restricted group was determined by the average food consumption of the chicks fed the deficient diet.

New Hampshire X White Leghorn cross-bred chicks 10 days of age at the start of the experiment were used. They were kept on experiment for 14-18 days and comparable numbers of deficient and control chicks were taken for histological study at the same time.

The effect of feeding the deficient diets on the growth rate was rapid and marked. The deficient chicks lost from 12 to 20 g during the experimental period, showed retarded growth and were emaciated. The chicks fed adequate levels of tryptophan gained an average of 124 g while the restricted group made an average gain of 24 g until the last 5 days of the experimental period. The chicks

of the latter group either lost weight or failed to gain during the last 5 days of the experiment due to the severe food restriction during this period. The hemoglobin and plasma protein levels for the deficient chicks were essentially normal after 14 days on experiment.

Histological observations were made in the following manner. The tissue was fixed in Bouin's fixative, dehydrated and imbedded in paraffin. Sections were prepared of 7 micron thickness. The slides were stained by each of the following methods: hematoxylin-eosin, Masson's trichrome technic and iron hematoxylin. Photomicrographs of sections from the control and tryptophan deficient chicks are shown in the accompanying figures.†

Histological examination of the tissues from the group receiving a restricted food intake demonstrated that the tissues were normal with the exception of the liver. The cells of the liver from the tryptophan deficient group appeared normal in size, shape and staining properties. The liver cells of the birds that received the restricted diet, however, showed a very lightly staining cytoplasm with large vacuoles present, which indicated that exhaustion of the cell contents occurred.

The following changes to be described were observed in chicks fed the tryptophan deficient diet. The gall bladder was greatly distended with thick mucous bile. The gall bladder itself revealed an erosion of the epithelium and mucosa with only fragments of these layers remaining intact. The perimuscular connective tissue formed the bulk of the gall bladder with an apparent decrease in the thickness of the smooth muscular layer. Difficulty was experienced in making accurate comparative measurements due to the degree of distention.

The spleen of the deficient chicks macroscopically appeared to be about one-fifth the size of the normal control. It was a grayish color instead of the normal dark red. Histologically the spleen contained very few erythrocytes and the splenocytes were irregular

† We are indebted to Paul Wilhelm for assistance in the preparation of slides and photomicrographs.

⁵ Maun, M. E., Cahill, W. M., and Davis, R. M., *Arch. Path.*, 1945, **39**, 294.

⁶ Maun, M. E., Cahill, W. M., and Davis, R. M., *Arch. Path.*, 1946, **41**, 25.

⁷ Wilkening, M. C., and Schweigert, B. S., *J. Biol. Chem.*, 1947, **171**, 209.

⁸ Wilkening, M. C., Schweigert, B. S., Pearson, P. B., and Sherwood, R. M., *J. Nutrition*, 1947, **34**, 701.

⁹ Schweigert, B. S., *Arch. Biochem.*, in press.

¹⁰ Toennies, G., *J. Biol. Chem.*, 1942, **145**, 667.

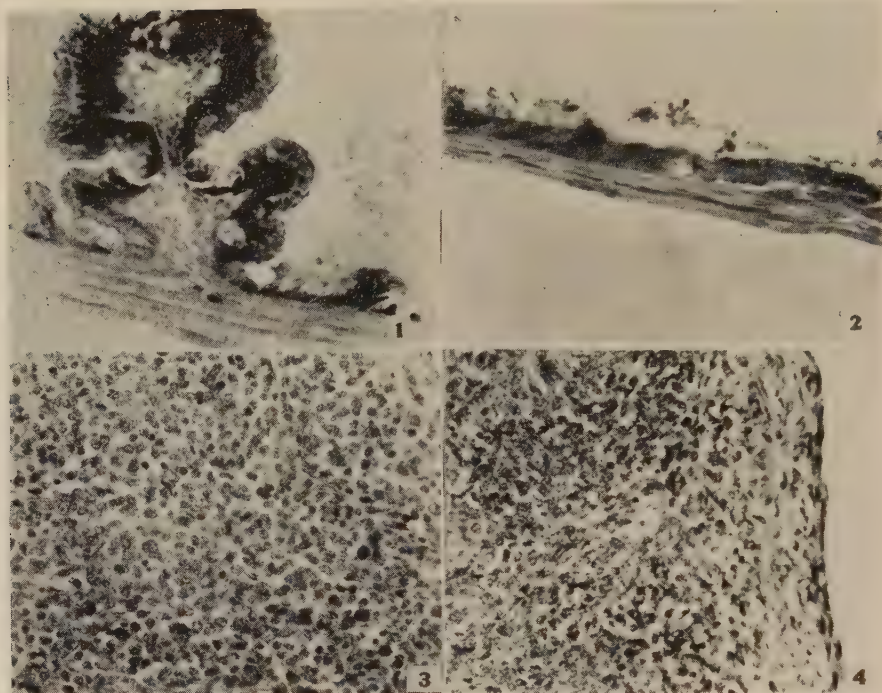


FIG. 1. Section of gall bladder of control chick showing development of the normal mucosa. ($\times 375$.)

FIG. 2. Section of gall bladder of tryptophan-deficient chick showing erosion of the mucosa. ($\times 375$.)

FIG. 3. Section of the normal spleen of the control chick. ($\times 375$.)

FIG. 4. Section of the spleen of a tryptophan-deficient chick showing degeneration and metachromasy of the reticulo-endothelial cells. The absence of erythrocytes may be noted. ($\times 375$.)

in size and showed signs of degeneration. Isolated splenic nodules were present.

The muscle tissue cells of the deficient chicks showed a decrease in diameter and staining property. The intercellular spaces were greatly increased so that most of the fibers remained as distinct entities. The spaces between the cells were evidently filled with a non-staining lymphoid fluid; hence such tissue could be considered as edematous. The average diameter of a muscle fiber from the control chicks was 21 microns as compared to 10.5 microns for muscle fibers from the deficient chicks.

The testes of both the control and the tryptophan-deficient birds were immature. The seminiferous tubules of the deficient chicks were larger in diameter and showed a degeneration of the seminiferous epithelium. The tubules lacked a central lumen and were ap-

parently filled with a lightly staining degenerative edematous mass. The nuclei of the spermatogonial cells were irregular; the interstitial cells were atrophic. The ovarian tissue showed a retardation in the development of the oocytes.

Discussion. The histological manifestations of this deficiency indicate what might be considered as 2 types. The first was apparently caused by the failure of the body to synthesize protein adequate for normal growth and was evidenced by a general exhaustion of cellular protoplasm as demonstrated by histological examination. This seems to be the case for such tissues as muscle and spleen. The second manifestation was evidenced by changes in the specific structure of the organ as in the degeneration of seminiferous tubules and the enlargement of the gall bladder and erosion of its layers.

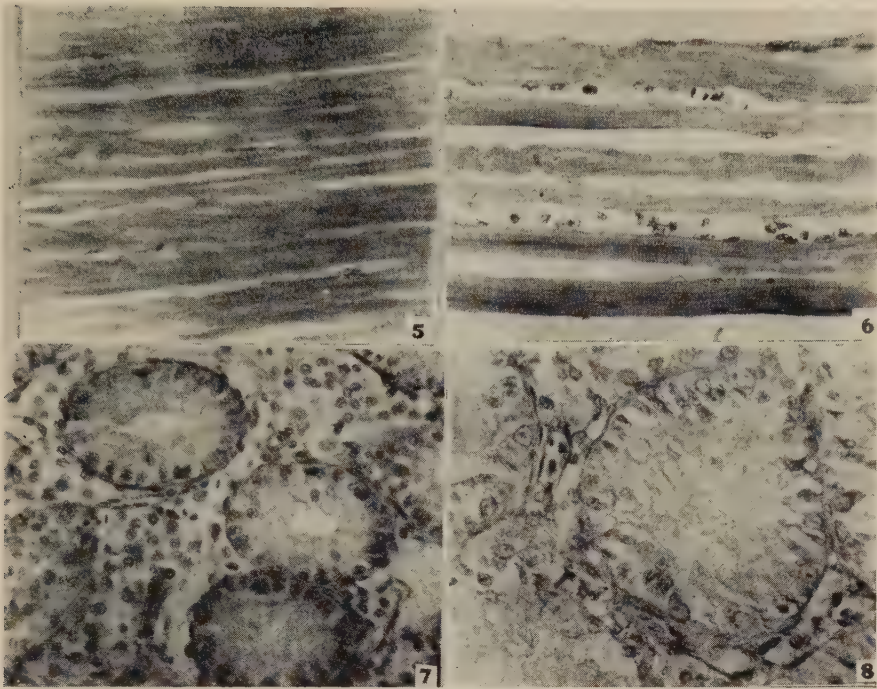


FIG. 5. Section of normal muscle tissue of the control. ($\times 375$.)

FIG. 6. Section of muscle tissue from tryptophan-deficient chick showing a decrease in diameter and separation of the muscle fibers. ($\times 375$.)

FIG. 7. Cross section of the immature seminiferous tubules of a control chick. ($\times 375$.)

FIG. 8. Cross section of the seminiferous tubules of a tryptophan-deficient chick showing degeneration of the tubular epithelium and swelling of the tubules and atrophy of the interstitial cells. ($\times 375$.)

Summary. Chicks fed a tryptophan deficient purified diet exhibited certain abnormalities when examined histologically. These were an enlargement and erosion of

the gall bladder, edematous swelling of the seminiferous tubules, degeneration of spleen cells and a decrease in the diameter of the muscle fibers.

Significance of Vitamin A Alcohol and Ester Partitioning under Normal and Pathologic Circumstances.*

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Previous studies¹ indicated that the low plasma vitamin A level in hepatic diseases and conditions associated with liver damage is at least in part due to disturbed release of vitamin A from the liver. On the basis of fluorescence microscopic observations,² this was explained by displacement and retention of vitamin A within the damaged liver cells. Recent investigations^{3,4} showed that vitamin A is transported in esterified form from the intestine to the liver where it is stored in this form.^{5,6} It is released from the liver to the blood as vitamin A alcohol.⁴ The latter represents about 80% of the total plasma vitamin A in the normal fasting human being and in several animals studied.^{3,4,5,6} In pathologic conditions, the total plasma vitamin A level may decrease, especially in liver diseases^{7,8} or it may be elevated as in recovery from these diseases⁹ or in renal altera-

tions.¹⁰ This raises the problem as to the changes of the vitamin A alcohol-ester partitioning in pathologic conditions.

Material and Methods. Total vitamin A and vitamin A esters were determined in the plasma of 186 fasting patients. In 9 instances the determinations were repeated 2, 3, 6, 24 and 48 hours after administration of vitamin A as non-saponifiable fraction of fish oil, dissolved in corn oil or in an aqueous dispersion.[†] The latter was prepared with sorbitan monolaurate derivative. In 5 cases, determinations were performed after 3 and 7 days on a diet containing not more than 20 units of vitamin A per day.

For the ester separation,¹¹ 4 cc of 95% ethyl alcohol were added to 4 cc of oxalated plasma and after mixing, 12 cc of petroleum ether were added. After shaking the mixture for 10 minutes, separation of the layers was permitted in a refrigerator. Then, a 10 cc aliquot of the petroleum ether was transferred to a separatory funnel and extracted seven times with 10 cc portions of 90% methyl alcohol, at a temperature between 10 and 30°C. The petroleum ether fraction was evaporated to dryness under nitrogen in vacuum. The residue was dissolved in chloroform and examined for vitamin A esters as in the total vitamin A determination according to the method of

* Supported by a grant from the Dr. Jerome D. Solomon Memorial Research Foundation and the Committee on Scientific Research of the American Medical Association, Chicago, Ill.

¹ Popper, H., Steigmann, F., Meyer, K. A., and Zevin, S., *Arch. Int. Med.*, 1943, **72**, 439.

² Popper, H., *Physiol. Rev.*, 1944, **24**, 205.

³ Clausen, S. W., McCoord, A. B., Baum, W. S., Steadman, L. G., Rydeen, J. O., and Breese, B. B., Abstract, Am. Chem. Soc., 100th Meeting, Detroit 47B, 1940.

⁴ Hoch, H., and Hoch, R., *Brit. J. Exp. Path.*, 1946, **27**, 316.

⁵ Baum, W. S., McCoord, A. B., Rydeen, J. O., and Breese, B. B., *J. Nutrition*, 1942, **24**, 1.

⁶ Glover, J., Goodwin, T. W., and Morton, R. A., *Biochem. J.*, 1947, **41**, 97.

⁷ Popper, H., and Steigmann, F., *J. A. M. A.*, 1943, **123**, 1108.

⁸ Adlersberg, D., Sobotka, H., and Bogatin, B., *Gastroenterology*, 1945, **4**, 164.

⁹ Steigmann, F., Meyer, K. A., and Popper, H., *Ann. Int. Med.*, 1945, **22**, 832.

¹⁰ Popper, H., Steigmann, F., and Dyniewicz, H. A., *Am. J. Clin. Path.*, 1945, **15**, 271.

[†] Supplied by Dr. Samuel M. Gordon of Endo Products, Inc.

¹¹ Gillam, A. E., and Senior, B. J., *Biochem. J.*, 1936, **30**, 1249.

¹² Kimble, M. S., *J. Lab. Clin. Med.*, 1939, **24**, 1055.

TABLE I.
Concentration of Total Vitamin A, Vitamin A Ester, Vitamin A Alcohol in μg per 100 cc Plasma and Vitamin A Ester Percentage of Fasting Hospital Controls and Patients Suffering from Various Diseases.

Diagnosis	No. of cases	Mean total Vit. A	Ester Vit. A			Alcohol Vit. A			Mean ester %
			Mean	Range	T value* compared with hospital control	Mean	Range	T value* compared with hospital control	
Hospital controls	31	51.2	9.5	3.0-15.1		41.7	25.1-78.0		18.2
Cardiac disease	13	38.7	9.5	2.3-16.8	0.03	29.2	9.0-47.7	2.71	26.8
Gastrointestinal disease	7	44.6	11.6	6.7-16.7	0.93	33.0	9.9-48.2	1.48	30.6
Carcinoma without involvement of biliary tract	15	34.8	13.1	2.3-21.0	2.07	21.7	6.1-54.3	4.54	42.4
Chronic wasting disease	5	29.3	10.3	2.3-21.6	0.32	19.0	5.1-52.5	3.37	39.5
Malnutrition	4	22.5	13.2	9.7-18.1	1.25	9.3	6.2-17.9	4.37	56.0
Tuberculosis	11	29.2	10.7	7.6-12.9	0.64	18.5	2.9-42.4	4.74	44.1
Infections	2	40.3	14.0	11.7-16.2	1.09	26.3	22.4-30.1	1.51	34.5
Pneumonia	9	27.4	14.6	10.0-20.0	2.38	12.8	2.8-24.4	5.47	53.4
Pneumonia recovering	10	53.2	12.7	2.1-29.6	1.59	40.5	14.1-64.5	0.23	24.1
Nephritis	10	67.3	11.1	3.3-17.4	0.81	56.2	31.7-105.8	2.87	17.3
Nephrosis	6	57.0	22.3	14.1-59.2	5.06	34.7	6.5-55.7	1.12	40.0
Acute infectious hepatitis	14	34.2	11.1	2.3-16.7	0.91	23.1	2.0-41.0	4.14	38.1
Infectious hepatitis recovering	6	40.8	12.0	8.3-12.4	1.02	28.8	10.9-57.0	2.07	32.4
Acute toxic hepatitis	6	26.3	12.9	7.8-18.9	1.36	13.4	6.7-21.7	4.34	46.4
Cirrhosis without jaundice	12	26.7	11.9	5.0-24.7	1.25	14.8	21.1-29.5	5.67	47.9
Cirrhosis with jaundice	17	23.1	12.0	4.0-24.7	1.51	11.1	1.7-27.9	7.25	56.9
Obstructive jaundice	8	39.4	11.6	5.0-21.5	0.94	27.8	11.6-65.0	2.50	31.9

* T test for significance of difference between means (see Fisher, R. A., *Statistical Methods for Research Workers*, Oliver and Boyd, Edinburgh, London, 1948). T value above 2.5 is considered significant.

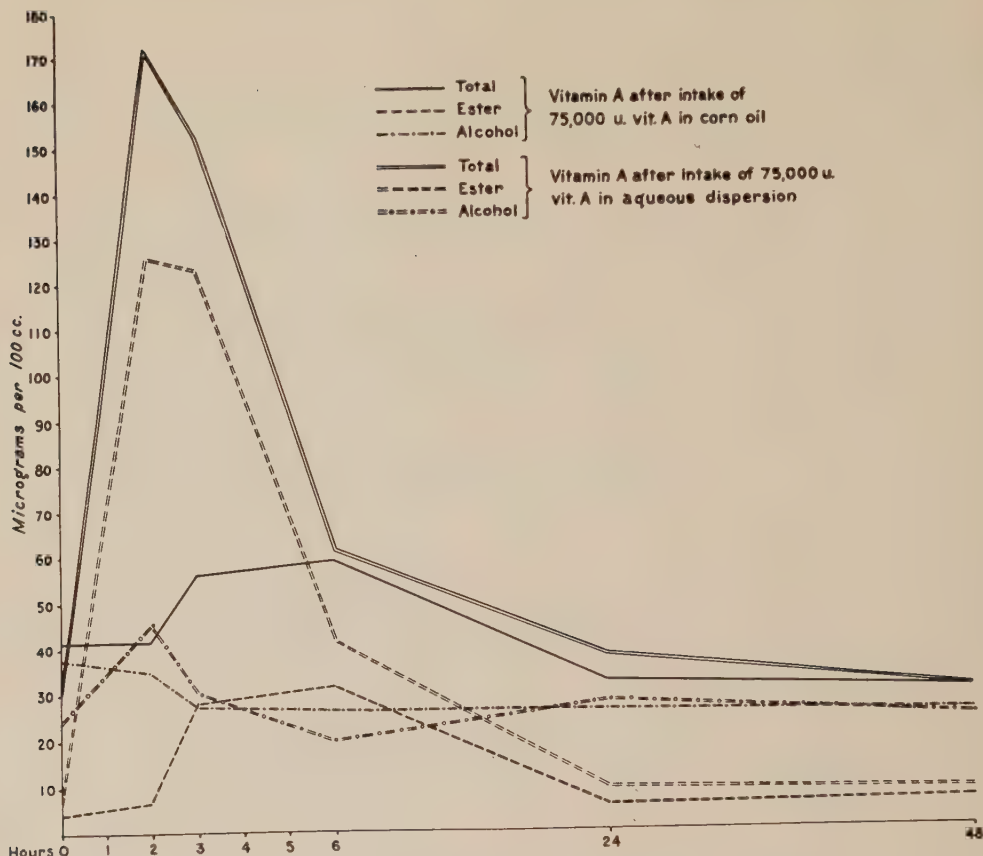


FIG. 1.

Response of total, ester, and alcohol vitamin A concentration in the plasma to the intake of 75,000 units of vitamin A in corn oil and in aqueous dispersion in a hospital control.

Kimble¹² using the Coleman spectrophotometer. The alcohol vitamin A was calculated by subtraction of the ester vitamin A from the total vitamin A concentration.

Results. In hospital controls, about 20% of the total vitamin A was found in the ester form (Table I). The average of the ester fraction revealed, under the examined pathologic circumstances, no statistically significant changes with the exception of the cases of nephrosis in which the ester vitamin A was significantly increased. In carcinoma (even without involvement of the biliary tract), malnutrition, pneumonia and especially in liver diseases, there was a definite trend for elevated ester vitamin A which was brought out by the average as well as by the maximal levels obtained.

The vitamin A alcohol revealed a statistically significant increase in cases of nephritis

in which the highest level of this series was recorded. In nephrosis, however, the level appeared on the average lower than normal without statistical significance. A similar, statistically not significant reduction was encountered in gastrointestinal diseases and in infections, whereas a significant reduction was found in cardiac diseases. In carcinoma without involvement of the biliary tract, in malnutrition, in severely sick persons, in tuberculosis and in pneumonia, the levels were low, the lowest of the entire group being found in pneumonia. The esters in these instances were high. After recovery from pneumonia the vitamin A alcohol returned to normal and might even exceed the normal. In acute, infectious or toxic hepatitis there was a significant reduction of vitamin A alcohol with irregular return to normal during recovery. In obstructive jaundice, the re-

TABLE II.

Mean Total Vitamin A, Vitamin A Ester, and Alcohol in μg per 100 cc Plasma and Ester Percentage of 5 Patients Kept on a Vitamin A-Deficient Diet.

Days on Vit. A deficient diet	Total Vit. A	Ester Vit. A	Alcohol Vit. A
0	42.8	11.1	31.7
3	38.2	12.4	24.2
7	31.4	13.9	17.9

duction was less significant. In cirrhosis, especially with jaundice, there was the most marked reduction of the plasma vitamin A level with the lowest values recorded. The esters in these instances were reduced, normal or elevated.

The peak of the vitamin A tolerance curve after feeding of 75,000 units of vitamin A varied in the different examined conditions. It was especially low in liver diseases following the intake of vitamin A in oil. However, the relation between alcohol and ester was the same in the examined normal and pathologic conditions in that the rise was entirely due to the ester fraction, whereas the vitamin A alcohol concentration showed no characteristic variations (Fig. 1). After intake of vitamin A in aqueous dispersion the rise of total and ester vitamin A was far higher than after administering the same amount of vitamin A in oil. During the peak of the tolerance curve the percentage of esters rose from around 20% to 80% in hospital controls.

The plasma vitamin A level dropped slightly in the patients kept for 7 days on a vitamin A deficient diet (Table II). This drop was due to a reduction of the alcohol fraction. The ester fraction dropped in one case slightly, whereas it rose in the other 4. In all instances, the percentage of the esters rose during the duration of the experiment.

Comment. This study confirms on a large number of subjects that in the fasting normal human being only about 1/5 of the total plasma vitamin A is in the esterified form. The ester portion increases after intake of vitamin A as well in aqueous as in oily menstruum in both normal and pathologic cases, while the alcohol does not change. Vitamin A esters are elevated in nephrosis in which cholesterol and other lipids are known to be increased. This elevation, therefore, may be

due to increased solubility in the blood. The ester fraction reveals a slight but not regular or statistically significant rise in malnutrition, carcinoma or liver diseases, while the total or alcohol vitamin A level is reduced. One could consider the ester rise in these latter conditions an indication of inability of the liver to absorb esters from the blood with their subsequent piling-up in the blood. However, vitamin A esters rise also while vitamin A is withheld from the diet. One might assume a higher threshold for vitamin A absorption in conditions associated with liver impairment as is the case with glucose and other metabolites. However, the fact that in such conditions the vitamin A ester level may be low militates against this possibility. No evidence is available for increased solubility of vitamin A. The fourth and so far most likely hypothesis would assume a pathologic release of vitamin A in ester form due to liver damage which prevents the hepatic esterase from converting vitamin A ester, the bulk of the stored hepatic vitamin A, into vitamin A alcohol. Only vitamin A alcohol present in small amounts in the liver was found to be in balance with the blood vitamin A alcohol,⁶ and is the form in which the liver releases vitamin A normally. With inactivation of the esterase and impaired release of alcohol, esters would enter the blood. This agrees with the observation that after administration of ethyl alcohol either the vitamin A ester⁵ or alcohol⁴ level may be elevated.

Vitamin A alcohol is increased in nephritis (not in nephrosis) and the reported high blood levels in this condition are due to the alcohol but are still not explained. The vitamin A alcohol also rises in recovery from pneumonia and hepatitis. The hypervitaminemia A in such conditions is probably the

result of increased release of vitamin A from the liver. Since the ester level is not significantly raised in this condition, the hyper-vitaminemia is apparently not, as it has been assumed,⁵ due to reduced uptake of vitamin A by the liver during the recovery period when the absorption has returned to normal but liver damage still persists. The fact that the blood vitamin A alcohol represents vitamin A released from the liver explains also its reduction in malnutrition, infections, hepatic and wasting diseases, *i.e.* conditions in which the hepatic vitamin A concentration is reduced as in chronic liver diseases or malnutrition, or vitamin A release is impaired as in acute diseases (pneumonia or hepatitis). This impairment was previously considered morphologically the result of a displacement of vitamin A in the liver cells² and now is believed, physiologically the result of impaired esterification.⁶

The reduction of the vitamin A alcohol concentration under pathologic conditions, often associated with a rise in esters, elevates the ester percentage like the intake of vitamin A, but obviously on an entirely different basis.

Although the total plasma vitamin A level

is shown to depend as much on endogenous⁷ as on nutritional factors, it is often used as a valuable index of general or vitamin A nutrition. However, the vitamin A alcohol level is a superior index because it reflects much better storage in and release from the liver and is independent of solubility and postprandial rise. Despite the more elaborate method of determination, the vitamin A alcohol level may replace the total plasma vitamin A level as a nutritional index.

Summary. Plasma vitamin A esters rise markedly after intake of vitamin A. They are elevated significantly in nephrosis and slightly and irregularly in conditions associated with hepatic impairment. The plasma vitamin A alcohol level does not change after the intake of vitamin A but is elevated in nephritis and sometimes in recovery from conditions associated with hypovitaminemia A. It is significantly decreased in malnutrition, infections, hepatic and wasting diseases, apparently due to reduced storage in and/or release from the liver. The vitamin A alcohol level, therefore, excels the total vitamin A level as index of hepatic vitamin A storage and vitamin A nutrition.

16592

Probability of Normal Development after Transplantation of Fertilized Rabbit Ova Stored at Different Temperatures.

M. C. CHANG. (Introduced by D. Rappoport.)

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In 1890 and 1897, Heape¹ reported his successful transplantation of rabbit ova to a uterine foster mother. This was mainly a demonstration of the unimportance of somatic tissue to the germ cells. Pincus and Enzmann² obtained normal young at term by

transplantation of rabbit ova fertilized or cultured *in vitro*. In my last communication,³ optimal temperature for the storage of ova and the normal development of young after low temperature storage of ova was reported. Since then, I have obtained the following data which show the probability of normal development.

Methods. The ova were obtained from

¹ Heape, W., *Proc. Roy. Soc.*, 1890, **48**, 457; 1897, **62**, 178.

² Pincus, G., and Enzmann, E. V., *Proc. Nat. Acad. Sc.*, 1934, **20**, 121.

³ Chang, M. C., *J. Gen. Physiol.*, 1948, **31**, 385.

TABLE I.
Probability of Development Following Transplantation of Fertilized Rabbit Ova, Recovered at Different Stages and Stored at Different Temperatures for Various Lengths of Time. F. Recovered from or transplanted into oviducts. U. Recovered from or transplanted into uterus.

Time of recovery after insemin.	Storage or culture		Recipient does		Total ova trans.		Fate of ova in pregnant does							
	Time, hr	Temp., °C	No.	Preg., %	No.	Normal devel., %	No. of ova	Normal devel., %	Degen. before implan- tation, %	Degen. after implan- tation, %	Sex of young			
											Male	Female	Dead	Total
25 F.	1-2	30	24 F.	91.6	239	54.4	209	62.2	30.6	7.2	61	60	9	130
72 F.U.	"	"	11 U.	45.5	132	31.1	57	71.9	24.6	3.5	13	15	12† + 1	41
96 U.	"	"	9 U.	55.6	89	23.6	43	48.8	48.8	2.3	11	10	0	21
25 F.	"	"	8* U.	12.5	109	.9	13	7.7	92.3	0	0	1	0	1
25 F.	"	"	6† U.	0	92	0	0	0	100.0	0	0	0	0	0
25 F.	24	38	13 F.	61.5	137	20.4	75	37.3	42.7	20.0	16	10	2	28
25 F.	48	"	9 F.	66.7	153	26.8	101	40.6	47.5	11.9	19	17	5	41
25 F.	72	"	6 F.	0	109	0	0	0	100.0	¶§	0	0	0	0
25 F.	24	10	7 F.	57.1	94	37.2	50	70.0	20.0	10	15	20	0	35
72 F.U.	24	"	7 U.	85.7	103	36.9	86	44.2	39.5	16.3	21	16	1	38
96 U.	24	"	8 U.	87.5	138	18.8	118	22.0	66.1	11.9	15	10	1	26
25 F.	96	"	14 F.	64.2	182	15.4	123	22.8	68.3	8.9	12	14	2	28
72 F.U.	96	"	4 U.	75.0	46	45.7	31	67.7	29.0	3.2	13	8	0	21
25 F.	24	0	7 F.	42.9	91	11.0	35	28.6	71.4	¶§	4	4	2†	10
25 F.	96	"	9 F.	44.4	146	6.2	56	16.1	82.1	1.8§	1	8	0	9
72 F.U.	96	"	4 U.	0	59	0	0	0	100.0	¶§	0	0	0	0

* Does ovulated 24 hr before operation.

† Does ovulated 72 hr before operation.

‡ Mother dead.

§ 1 or 2 animals diagnosed as pregnant at 2 weeks.

|| Abnormal young.

superovulated rabbits⁴ and transplanted within 1 to 2 hours or stored in undiluted rabbit serum at 0° C (cooled down in 5 hours) or at 10° C, or cultured at 38° C (subculture every 24 hours) for various lengths of time and then transplanted. The recipient does were ovulated by injection of gonadotrophin 1, 3, and 4 days before the operation according to the stage of ova recovered, irrespective of the time interval of storage or culture. Ova recovered from oviducts 25 hours after insemination (mostly in the 2 cell stage) were transplanted into oviducts through fimbria, while those recovered 72 hours (mostly in the morula stage, some in the oviducts and some in the uteri) and 96 hours after insemination (some in the blastocyst stage, nearly all in uteri) were transplanted into uteri by puncturing the uterus with a pipette containing ova. In 2 series of experiments, the ova at 2 cell stage were transplanted into the uteri of recipient does who had been ovulated 1 or 3 days before operation to determine receptibility of uterus to the ova at early stage. In each case 5 to 20 ova were transplanted depending on the time interval of storage, *i.e.*, more ova if stored for a longer time. Most of the pregnant recipient does were killed 25 to 28 days after operation. The number of normal young, the number of dead fetus in normal form (considered as normal development), the number of fetal placenta (absorption of embryo or fetus), and the number of maternal placenta (ova implanted but failing to develop) were counted.

Results. The complete results are presented in the accompanying table. The following facts are apparent from the data: (1) Of the total ova freshly transplanted, the percentage of normal development decreases from 54.4 to 23.6 when the ova were recovered from 25 to 96 hours after insemination. Thus, the younger the ova, the better the chance for normal development. (2) When the ova were cultured at 38° C for 24 or 48 hrs., the percentage of normal development is 20.4 or 26.8 respectively, whereas for 72 hours, no young were obtained. (3) For storage

at 10° C for 24 hours, the percentage of development of the total ova transplanted is 37.2, 36.9 and 18.8, when the ova were recovered 25, 72, and 96 hours after insemination. Considering the percentages of development in the pregnant does (70, 44.2, and 22), it is again demonstrated that the younger the ova the better the chance for survival after storage. This percentage is 15.4 or 45.7 after storage at 10° C for 4 days with the ova recovered 25 or 72 hours after insemination. The latter percentage is exceptionally high, perhaps due to the small number of cases. (4) At 0° C, the percentage of normal development is very low even when stored for 24 hours (11%). But normal young were obtained after storage for 4 days at 0° C (6%). It seems, therefore, that 10° C is the optimal temperature for storage of ova as determined in a previous study on cleavage *in vitro*.³ (5) It is interesting to note that even freshly recovered 2-cell ova cannot survive as embryos in the uterus regardless of whether the recipient does were ovulated 24 or 72 hours before transplantation. It seems that the 2-cell ova can survive *in vitro* at 0°, 10°, or 38° but fail *in utero*. Although the chance of survival *in vitro* is better in the case of ova recovered early, the results clearly show that only ova developed to a certain stage can survive and develop *in utero*.

The percentage of ova recovered, as checked by the number of ovulation points in the ovaries, was about 90% from oviducts. It was about 40% from the uterus 72 hours after insemination, but about 65% from uterus 96 hours after insemination. The sex ratio of the offspring was not altered after storage of ova *in vitro*; therefore, there is no obvious differential mortality of male or female zygotes at different temperatures *in vitro*. Only one abnormal young (hernia and deformation of fore limbs) was obtained in this investigation, but indications of degenerated embryos were observed in each treatment. It is highly probable that the defective ova or embryos were degenerated and absorbed rather than developed into abnormal young.

⁴ Pincus, G., *Anat. Rec.*, 1940, **77**, 1.

The percentage of ova degenerated at dif-

ferent stages in the pregnant recipients as recorded in the table may be lower than what happened in all the recipient animals because all non-pregnant does were not sacrificed for examination. Several animals were determined as pregnant by palpation 2 weeks after operation but found to be not pregnant at a later stage, presumably due to the early degeneration of embryos. In general, the percentage of ova failing to implant is higher when the ova were recovered at a late stage and/or when they were stored or cultured. The percentage of degenerated embryos or fetus is higher after storage or culture of ova (8.9 to 20%) as compared with fresh transplantation (2.3 to 7.2%).

The large number of ova produced by gonadotrophin administration are capable of developing into normal young as is demonstrated in the following cases: In one case, 53 two cell ova recovered from one doe were freshly transplanted into 4 does and 45 normal young were obtained. In another case, 26 young were obtained from 3 recipients by the transplantation of 35 two cell ova from one doe.

Discussion. In view of the fact that, in the rabbit, about 33% of ova degenerate⁵ in the course of development following natural mating, the effectiveness of transplantation of freshly recovered two cell ova (54.4 to 62.2%) is nearly similar to the natural course. While the transplantation of two cell

ova after 24 hours at 10°C (37.2 to 70%) is about 15% lower, and that of morulae and blastocysts is about 30% lower than the natural course.

For the full utilization of the germ cells of valuable animals, there is a great difference between the spermatozoa which are produced by the thousands of millions in one ejaculation and the ova which are only one or two in number per heat period. However, considering the fact that one ovum requires millions of spermatozoa to insure fertilization and the fact that the number of ova ovulated can be much increased by the administration of gonadotrophins, it seems that there may be practical value in developing the technic of ovum transplantation and related fundamental research for animal industry.

Summary. The percentages of fertilized rabbit ova developed into normal young after transplantation of the ova recovered at different stages (from 2-cell ova to blastocysts), and stored at different temperatures (0°, 10°, 38° C) for various length of time (24 to 96 hours) into the oviducts or uteri of recipient does were reported.

The writer wishes to acknowledge his gratitude to Dr. Hudson Hoagland and Dr. Gregory Pincus for giving him the opportunity to work in their laboratory and especially to Dr. Pincus for advice. Thanks are also due to Foundation for Applied Research, San Antonio, Texas, for a grant and to Mr. Raymond A. Gunnerson for assistance.

⁵ Hammond, J., *Z. f. Pelztier- und Rauchwarenkunde*, 1931, **3**, 56.

Saline and Methionine-Saline Effects on Survival Rate of Rats Receiving Standardized Burn Shock.*

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McCarthy and Bodkin¹ have reported that the oral administration of 160 mg of methionine to rats, 2 hours before a standard thermal injury significantly increased the 48-hour survival rate. Methionine administered at varying periods following thermal injury failed to influence the survival. In 1945 we investigated the effect of methionine and saline in rats receiving standardized burns. Due to unavoidable circumstances, these experiments were interrupted, and only recently have they been completed. The methionine effects are reported here as largely confirmatory to the data of McCarthy and Bodkin.¹

Experimental. Adult rats of the Sprague-Dawley and Wistar strains were used. Previous to the experimental procedure, the animals received a commercial stock ration and distilled water *ad libitum*. The standard burn was produced by the technic of Rosenthal,² modified as described below. The water bath was maintained within $\pm 0.5^\circ$ of the desired temperature and the duration of the immersion was determined with a stopwatch. The animals were not shaven and were completely immersed to the center of the neck region. Fifteen minutes before immersion 0.1 cc of sodium pentobarbital solution (6 mg) per 100 g body weight was injected intraperitoneally. In preliminary experiments pentobarbital appeared to be superior to ether in that a more uniform depth of anesthesia was obtained and the anesthesia persisted for a longer time following the burn. Immediately after immersion, the animals were wrapped

in a dry towel to remove excess water and then placed in individual cages with access to food and water. They were observed at 5 hour intervals for 50 hours. Animals dying during a 5 hour period were recorded as dying at the end of that period. The experimental animals received by intraperitoneal injection, 2.50 or 1.25 cc of a 1.5% solution of methionine in isotonic saline per 100 g of body weight. The controls received comparable volumes of saline. We found the results of this simplified method of producing a standard burn to be highly reproducible from day to day.

Results. The data are summarized in the table. Apparent differences were analyzed statistically and only those in which the difference was 3 or more times the standard error of the differences were considered significant. All animals injected one hour before immersing for 15 sec. at 70° C survived. Next, the temperature was elevated to 75° , with the immersion period remaining at 15 sec. The 5° elevation in temperature was sufficient to decrease the survival rate of the animals injected with saline one hour before burning from 100.0 to 33.3%. The methionine under these conditions gave a highly significant increase in the survival rate, increasing the rate from 33.3 to 91.7%. In the experiments of McCarthy and Bodkin¹ the animals received approximately 80 mg of methionine per 100 g body weight, whereas in the present study 37.5 mg per 100 g produced a significant effect. These results confirm the findings of McCarthy and Bodkin.¹ A comparison of the uninjected controls with those receiving saline one hour before burning indicates that the saline was without effect. Prinzmetal and co-workers³ have reported that

* Preliminary experiments were carried out by the junior author in the Biochemistry Laboratory, Southwestern Medical College, Dallas.

¹ McCarthy, M. D., and Bodkin, R. E., *Proc. Soc. Exp. Biol. and Med.*, 1946, **63**, 377.

² Rosenthal, S. M., *U. S. Pub. Health Rep.*, 1942, **57**, 1923.

³ Prinzmetal, M., Hechter, O., Margolis, C., and Feigen, G., *J. Am. Med. Assn.*, 1943, **122**, 720.

TABLE I.
Effect of Saline and Methionine-Saline Injections on the 50-Hour Survival Rate of Rats Receiving Standardized Burn Shock.

Bath temperature, °C	Duration of immersion, sec.	Injection			Survival, %
		No. of rats	Solution*	Volume,† cc	
75	15	Uninjected controls.			
		22			13.6
		Injected 1 hour before burning.			
70	15	12	S	2.5	100.0
70	15	12	MS	2.5	100.0
75	15	12	S	2.5	33.3
75	15	12	MS	2.5	91.7
		Injected at time of burning.			
75	15	11	S	2.5	72.7
75	15	11	MS	2.5	72.7
75	20	8	S	2.5	62.5
80	15	8	S	2.5	50.0
80	15	22	S	1.25	13.6
80	15	21	MS	1.25	28.6
		Injected 1 hour after burning.			
75	15	34	S	2.5	79.4
75	15	35	MS	2.5	68.6
75	15	19	S	1.25	26.3
75	15	22	MS	1.25	13.6
80	15	12	S	2.5	58.3
80	15	12	MS	2.5	41.7

* S signifies isotonic saline; MS, methionine-saline.

† Volume injected per 100 g body weight.

the administration of isotonic saline in amounts equivalent to 5% of the body weight one-half hour before burn shock significantly increases the survival time. In the animals injected at the time of the burn (75° - 15 sec), the saline was highly protective, increasing the survival rate from 13.6 to 72.7%. The methionine-saline solution gave the same survival rate as saline alone under these conditions. Because of the protective effect of saline alone, the experimental conditions were not satisfactory for demonstration of an effect of methionine, so the conditions were varied to produce a lower survival rate in the saline-injected animals. The best conditions found were 80° C for 15 sec. with injections of 1.25 per 100 g body weight. However, the 28.6% survival rate of the methionine-saline treated rats was not significantly different from the 13.6% observed in the saline controls. Therefore, it was concluded that while isotonic saline was highly protective against burn shock when administered at the time of the burn, the addition of methionine was without effect. The effect of the two solutions one hour after burning was determined

under 3 different sets of conditions. In all 3 experiments the methionine-saline solution gave a slightly lower survival rate than the saline alone. However, the differences were not statistically significant. The saline solution was as effective when given one hour after the burn as when given at the time of the burn. Rosenthal⁴ has previously reported that isotonic saline lowers the mortality when administered one or more hours following a standard burn. Croft and Peters⁵ have shown that methionine has a protein-sparing action when fed to rats following thermal burns.

The explanation for the difference in effect of methionine when given before a burn and at the time or after a burn is not apparent. It is possible that the action of methionine involves preliminary processes in the liver or other organs which are interfered with when the methionine is administered at a later time in relation to the burn. Also, these results do not preclude the possibility that larger

⁴ Rosenthal, S. M., *U. S. Pub. Health Rep.*, 1943, **58**, 513.

⁵ Croft, P. B., and Peters, R. H., *Lancet*, 1945, **1**, 266.

amounts or repeated doses of methionine might increase the survival rate.

Summary. A simple and convenient method for producing standardized burn shock in rats is described. Isotonic saline injected intraperitoneally one hour before a standardized burn shock did not increase the

survival rate; administration at the time of the burn or one hour following significantly increased the survival rate. Methionine-saline solution given one hour before burning significantly increased the survival rate; given at the time of the burn or one hour following, the effect was the same as with saline alone.

16594

Enhancement of Epinephrine Induced Cardiac Arrhythmias by Tetraethylammonium Chloride (Etamon).*

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In the experiments to be reported herein tetraethylammonium chloride has been shown to intensify the cardiac arrhythmias occurring in dogs during cyclopropane anesthesia. Secondly, experiments on unanesthetized animals suggest that the choice of a pressor agent for use as an antidote after tetraethylammonium chloride may be critical. After tetraethylammonium injection epinephrine resulted in ventricular tachycardia whereas phenylephrine (Neo-Synephrine) caused no arrhythmias.

Cardiac arrhythmias occurring during cyclopropane anesthesia have been demonstrated experimentally to depend upon reflex sensitization of the heart to epinephrine.¹ Cyclopropane stimulates receptors located in the abdominal viscera, producing afferent impulses which pass to the cord by way of fibers travelling with the splanchnics. The reflex center is located in the brain, presumably in the hypothalamus. Efferent impulses

pass to the heart by way of cardiac sympathetics. Since interruption of this pathway at any point has prevented the arrhythmias, the tetraethylammonium ion should theoretically block the reflex sensitization. This agent has been reported to interrupt sympathetic impulses by ganglionic blockade² and consequently was expected to block the efferent impulses.

When it was found that tetraethylammonium potentiated the cardiac effects of epinephrine under cyclopropane, the cardiac effects of the ion and epinephrine were also investigated in unanesthetized animals. Epinephrine has been recommended as an antidote for the profound hypotension which may follow the clinical administration of tetraethylammonium.³

Cyclopropane-epinephrine ventricular tachycardia was produced in the dog by the method described by Meek *et al.*⁴ The only modification of this technic was reduction of the standard test dose of epinephrine in order to lower the mortality from ventricular fibrillation. After the control duration of ventricu-

* Aided by a grant from the Committee on Scientific Research of the American Medical Association.

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¹ (a) Allen, C. R., Stutzman, J. W., and Meek, W. J., *Anesthesiology*, 1940, **1**, 158; (b) Stutzman, J. W., Murphy, Q., Allen, C. R., and Meek, W. J., *Anesthesiology*, 1947, **8**, 579; (c) Pettinga, F. L., and Stutzman, J. W., *Fed. Proc.*, 1948, **7**, 93.

² Acheson, G. H., and Moe, G. K., *J. Pharmacol. and Exp. Therap.*, 1946, **87**, 220.

³ Berry, R. L., Campbell, K. N., Lyons, R. H., Moe, G. K., and Sutler, M. L., *Surgery*, 1946, **20**, 525.

⁴ Meek, W. J., Hathaway, H. R., and Orth, O. S., *J. Pharmacol. and Exp. Therap.*, 1937, **61**, 240.

TABLE I. Effect of Tetraethylammonium Chloride on Cyclopropane-Epinephrine Cardiac Arrhythmias in 15 Dogs.

Cardiac response to epinephrine	Before tetraethylammonium			After tetraethylammonium		
	No.* of animals	Duration of arrhythmia, sec		Dose, mg/kg	No.* of animals	
		Avg	Range		Avg	Range
VT	2	22.5	20-25	—	300	300
VF	—	—	—	20	2	—
VT	6	47	1-90	10	6	55
VF	—	—	—	10	2	5-100
F VPC	3	15	10-20	—	—	—
M VPC	2	11.5	3-20	10	1	30
VT	5	70	25-95	5	4	65
VF	—	—	—	5	1	25-95
						207-325
						—

VT = Ventricular Tachycardia; VF = Ventricular Fibrillation.

F VPC = Few Ventricular Premature Contractions (Less than 1 VPC to 6 supraventricular beats).

M VPC = Many Ventricular Premature Contractions (More than 1 VPC to 6 supraventricular beats).

Note: The concentration of cyclopropane was controlled at 30% in oxygen. These animals were in Plane 3 to 4 of Stage III.

* Since certain animals had more than one type of arrhythmia total incidence in these columns is in excess of total number of animals which was 15.

TABLE II.

Effect of Tetraethylammonium Chloride on the Cardiac Response to Epinephrine in 20 Unanesthetized Dogs.						
Cardiac response to epinephrine	Before tetraethylammonium			After tetraethylammonium chloride 10 mg/kg		
	No. of animals*	Duration of arrhythmia in sec.		No. of animals*	Duration of arrhythmia in sec.	
		Avg	Range		Avg	Range
SA Brady.	3	—	—	1	—	—
AV N Brady.	3	—	—	—	—	—
F VPC	8	29	10-45	—	—	—
M VPC	1	20	—	6	46	30-75
VR	5	22	10-55	—	—	—
Bigem	—	—	—	9	43	15-70
VT	4	9	3-15	19	78	25-160
						125
						—
						—
						210
						136-250
						—
						170
						125-215
						296
						214-375

SA Brady. = Sino-auricular bradycardia; AV N Brady. = Auriculo-ventricular nodal bradycardia; F VPC = Few ventricular premature contractions (less than 1 VPC to 6 supraventricular beats); M VPC = Many ventricular premature contractions (more than 1 VPC to 6 supraventricular beats); Bigem = Bigeminal rhythm (SA-V); VR = Ventricular rhythm; VT = Ventricular tachycardia.

* Since certain animals showed more than one type of arrhythmia the total incidence in these columns is in excess of the total number of animals which was 20.

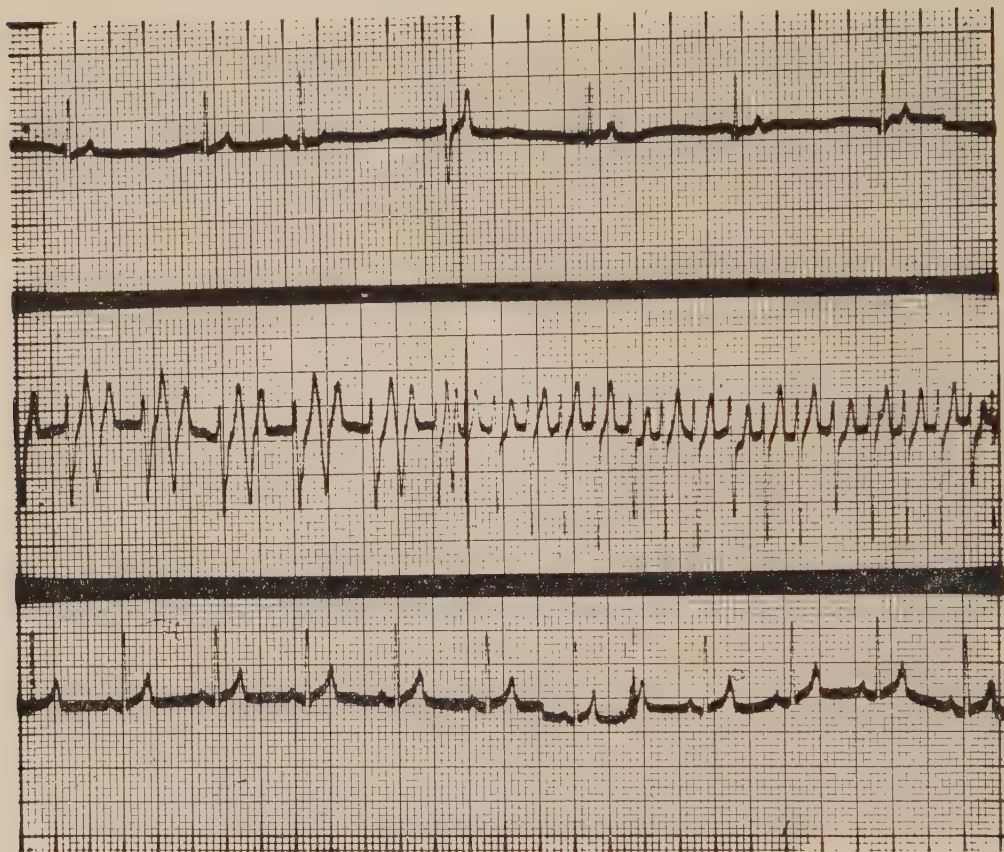


FIG. 1.

All electrocardiograms are from the same unanesthetized dog.

Upper tracing: Response to intravenous injection of 0.01 mg/kg of epinephrine; interpreted as auriculo-ventricular nodal rhythm with 1 sino-auricular and 1 ventricular premature contraction.

Middle tracing: Response to same dose of epinephrine 5 minutes after the intravenous administration of 10 mg/kg of tetraethylammonium chloride; interpreted as ventricular tachycardia.

Lower tracing: Response to the intravenous injection of 0.05 mg/kg of phenylephrine 5 minutes after 10 mg/kg of tetraethylammonium chloride on a subsequent day; interpreted as sino-auricular rhythm.

lar tachycardia had been determined, tetraethylammonium chloride[§] was administered intravenously over a period of 60 seconds in doses of 5 to 20 mg/kg. Ten minutes later the test dose of epinephrine[§] was repeated. The results of experiments on 15 animals are

[§] Tetraethylammonium chloride (Etamon) and epinephrine (Adrenalin HCl) were kindly furnished by Parke, Davis & Co., Detroit; phenylephrine HCl (Neo-Synephrine) by the Department of Medical Research, Winthrop-Stearns, Inc., Boston; and pentobarbital sodium by Eli Lilly & Co., Indianapolis.

summarized in Table I. Tetraethylammonium not only afforded no protection in any dosage tested but with doses of 10 or more mg/kg actually increased the duration of epinephrine induced ventricular arrhythmias. Bilateral supradiaphragmatic splanchnicectomy and sympathetic chain section at T-10 were performed on 4 dogs and prevented cyclopropane-epinephrine ventricular tachycardia by interrupting the afferent limb of the reflex arc. In these animals 10 mg/kg of tetraethylammonium chloride followed in 10 minutes by epinephrine resulted in ventricular tachy-

cardia of longer duration than the control. This dose of tetraethylammonium was found to be effective in blocking the cardiac acceleration from electrical stimulation of the preganglionic fibers to the stellate.

Since the above experiments suggested that tetraethylammonium was sensitizing the heart to epinephrine in spite of effective sympathetic block, the response was studied on 20 unanesthetized dogs. Epinephrine in doses of 0.003 to 0.01 mg/kg was administered intravenously at a constant rate over 50 seconds. The effects on cardiac rhythm were determined by electrocardiographic tracings (Lead II). The results of epinephrine before and 5 minutes after 10 mg/kg tetraethylammonium chloride are compared in Table II. In 19 of the 20 animals the cardiac arrhythmias were definitely more severe and of longer duration after tetraethylammonium. In 10 animals the response to phenylephrine hydrochloride (Neo-Synephrine)⁵ after tetraethylammonium was tested. In each case the sino-auricular node remained the pacemaker, and the only electrocardiographic evidence of phenylephrine injection was a slight decrease in rate. Fig. 1 shows electrocardiograms from a typical experiment.^{||} The dosage of phenylephrine was 0.05 mg/kg which has a pressor effect equivalent to 0.01 mg/kg of epinephrine in unpremedicated dogs.

The mechanism by which the tetraethylammonium ion potentiates the cardiac effects of epinephrine has not been elucidated. That the arrhythmias are simply the result of epinephrine stimulating a heart freed of vagal control is unlikely in view of the work of Wilburne, Surtshin, Rodbard and Katz who reported that atropine prevented ventricular

tachycardia resulting from injection of large doses of epinephrine in the unanesthetized dog.⁵

Corcoran and Page reported that tetraethylammonium chloride potentiated the pressor effects of epinephrine in the unanesthetized dog.⁶ In extending the pressor potentiation observation in animals anesthetized with pentobarbital Page and Taylor⁷ warned of the possibility that a safe antidotal dose of epinephrine for pressor effect may be much less than usual after tetraethylammonium has been administered. In the present investigation it was found in 6 dogs that the maximum level of mean blood pressure with phenylephrine was not altered significantly by previous injection of tetraethylammonium chloride. The total change in blood pressure was greater with phenylephrine after tetraethylammonium, but this was the result of hypotension from tetraethylammonium. Blood pressure was recorded by cannulation of the femoral artery under local anesthesia in lightly morphinized dogs and also in animals anesthetized with pentobarbital sodium.⁸

From the results of our experiments it is concluded that:

1. Epinephrine is contraindicated as a pressor agent after tetraethylammonium chloride.
2. Phenylephrine (Neo-Synephrine) is a satisfactory pressor agent after tetraethylammonium chloride.
3. Tetraethylammonium chloride is contraindicated for the prophylaxis or treatment of cardiac arrhythmias during cyclopropane anesthesia.

^{||} Electrocardiograms were recorded with a Sanborn Viso-Cardiette made available through the kindness of Mr. J. L. Jenks, Jr., of the Sanborn Co., Cambridge, Mass.

⁵ Wilburne, M., Surtshin, A., Rodbard, S., and Katz, L. N., *Am. Heart J.*, 1947, **34**, 860.

⁶ Corcoran, A. C., and Page, I. H., *Proc. Soc. Exp. Biol. and Med.*, 1947, **66**, 148.

⁷ Page, I. H., and Taylor, R. D., *J. A. M. A.*, 1947, **135**, 348.

Society for Experimental Biology and Medicine

Annual Report of the Secretary

July, 1948

The Council and the National Committees transacted the business of the year in Atlantic City, March 16-17, 1948.

The following is a brief statement of decisions reached:

Finances and related matters.

Fiscal year changed. The Council and National Committee transact their business during the Federation meetings. Such meetings occur usually in mid-March. Our fiscal year ends March 31. It was therefore decided, beginning this year, to change our fiscal year to end February 28. Hence the Treasurer's report, given below, is for 11 months only, April 1, 1947-February 29, 1948.

Deficit. The Treasurer's report below, shows a deficit of \$4705.81 for these 11 months. This deficit is due almost exclusively to increased cost of printing the PROCEEDINGS.

Increased sources of income. To meet these increasing costs and to anticipate further increases, the Council authorized the following changes.

1. *Increase members' dues* from \$5.00 to \$6.50 a year beginning September, 1948.

2. *Increase subscriptions* from \$8.50 to \$10.00.

3. *Advertising.* Suitable advertising may be accepted. A committee was appointed to report on policies. This committee consisted of Drs. G. R. Cowgill (Chairman), A. J. Goldforb (ex officio), G. M. Nelson, and H. B. Van Dyke. Their report was approved. This committee is now a standing committee to select an expert solicitor of advertising, to pass on all proposed advertising and rates. The committee will serve three years in rotation.

4. Another committee was appointed to report on the desirability of inviting persons and companies to contribute towards the cost of the PROCEEDINGS. Drs. H. T. Clarke (Chairman), H. S. Gasser, A. N. Richards and J. A. Shannon were appointed. They recommended (a) that such policy was inadvisable, (b) that the costs of publication should be met by an increase in dues and subscriptions, (see above), (c) that members be invited to pay voluntarily

\$10.00 a year and be listed as sustaining members. Their report was approved.

The PROCEEDINGS and Editorial Matters

There has been a progressive increase in the number of manuscripts accepted by the editors for the PROCEEDINGS. For October 1947 to June 1948, the number of manuscripts so far approved for publication is 542 of which 79% were "complete," 21% preliminary.

Delays in publication. These may be summarized as follows:

1. *Printer's delays.* It is hoped that these may subsequently be reduced.

2. *Delays due to increased number of pages printed for each issue.* (See below.)

3. *Delays in mailing, by postal offices, institutional offices and by members.*

4. *Illness or absence of editors.*

5. *Authors and sponsors who do not conform to "Instructions to Authors" on the cover of each PROCEEDINGS; authors and sponsors who do not reasonably meet the suggestions of the editors, with consequent repeated correspondence.* Luckily the number of members in this group is small.

We are making every effort to reduce these delays. The cooperation of authors, sponsors, and Section officers is requested.

Extra Issue of the PROCEEDINGS. The number of accepted manuscripts for the June and the October numbers is about twice that of other issues. Such large issues mean considerable delay in publication. In spite of the increased cost, and to reduce such delays, the Council approved the publication of an extra issue to be known as the July-August number.

Editors. I sometimes question whether our members fully realize the heavy burden on the editors, their devotion, their desire to help authors. I wish to express to each of them the full appreciation of their efforts, and the thanks of the Council.

Rotation of Editors. In conformity with our policy of rotation after 5 years of service, Drs. C. A. Dragstedt and J. Sendroy, Jr., both of whom gave very exceptional service, were succeeded by Drs. Earl Loew and Dean Burk.

Dr. Shields Warren was elected editor in Pathology to succeed Dr. Balduin Lucké. Dr. Oscar Wintersteiner was elected additional editor in Chemistry.

National Membership Committee. It is a pleasure to announce that under the revised procedures, the time required to consummate election to membership was reduced to 63 days. Out of 144 applications 125 were approved and applicants promptly notified.

Dr. H. C. Hodge, of the University of Rochester Medical School, Rochester, N. Y., was elected secretary of this committee. Hereafter all communications concerning applications for membership should be addressed to him.

The Council approved 7 requests for transfer to emeritus membership; 16 resignations. Four members were dropped for arrears.

Peiping Section. We are happy to announce that the Peiping Section is now functioning again.

Baltimore Section. The Council approved

the formation of this new Section.

The Council extends its deep appreciation and thanks to Dr. Emil Baumann of Montefiore Hospital, N. Y., for the very efficient indexing of the PROCEEDINGS.

to Mr. H. G. Friedman for his very expert and ever watchful care of our investments,

to Mr. Leon Leighton for continuous legal advice in matters pertaining to our mortgage investments, and to

Paul A. Zahl who directed a study of the publication activity of our members, by states and Sections. This report will be made available in the near future.

Deaths. The Council records with sorrow the death of the following members: J. C. Abels, J. Auer, E. J. Carey, D. R. Climenko, A. G. Cole, C. A. Elsborg, T. C. Evans, G. O. Favorite, C. B. Freudenberger, R. G. Green, Reid Hunt, F. C. Koeh, C. A. Kofoid, F. R. Lillie, J. H. Musser, R. N. Nye, Max Pinner, G. B. Wallace, and E. J. Witzemann.

SECTIONAL MEETINGS AND MEMBERSHIP

Baltimore

Chairman: W. R. Amberson Secretary: H. R. Himwich Members: 61

Cleveland, Ohio

Chairman: R. Heinle Secretary: W. H. Pritchard Members: 58

Meetings: Western Reserve University, October 10, 1947
November 14, 1947
December 12, 1947
January 9, 1948
February 13, 1948
March 12, 1948
April 9, 1948
May 14, 1948

District of Columbia

Chairman: H. R. Bird Secretary: D. Burk Members: 108

Meetings: George Washington University, December 4, 1947
February 5, 1948
April 1, 1948

Illinois

Chairman: J. Gray Secretary: H. Necheles Members: 232

Meetings: Abbott Laboratories, October 22, 1947
University of Chicago, December 9, 1947
University of Illinois, February 10, 1948
University of Illinois, April 13, 1948
Michael Reese Hospital, May 25, 1948

SECRETARY'S REPORT

Iowa

Chairman: W. O. Nelson Secretary: S. B. Barker Members: 47

Minnesota

Chairman: C. F. Code Secretary: J. M. Adams Members: 82

Meetings: University of Minnesota, October 22, 1947

Mayo Foundation, November 14, 1947

University of Minnesota, December 10, 1947

February 11, 1948

March 10, 1948

April 16, 1948

May 12, 1948

Missouri

Chairman: L. R. Jones Secretary: C. F. Cori Members: 62

New York

Chairman: E. Shorr Secretary: M. Levy Members: 532

Meetings: New York Academy of Medicine, December 10, 1947

January 21, 1948

Rockefeller Institute, February 25, 1948

New York Academy of Medicine, May 5, 1948

Pacific Coast

Chairman: M. Kleiber Secretary: J. J. Eiler Members: 140

Meetings: University of California, September 13, 1947

Stanford University, October 29, 1947

University of California, December 10, 1947

Permanente Foundation, February 25, 1948

Stanford University, April 17, 1948

Peiping, China

Chairman: S. H. Zia Secretary: S. C. Liu Members: 30

Meetings: Peiping Union Medical College, May 1, 1948

Rocky Mountain

Chairman: I. E. Wallin Secretary: R. M. Mulligan Members: 46

Meetings: Colorado A. and M. College, October 18, 1947

University of Colorado, February 21, 1948

Fort Logan, Colo., May 22, 1948

Southern

Chairman: M. F. Shaffer Secretary: F. G. Brazda Members: 61

Meetings: Tulane University, November 7, 1947

Birmingham, Alabama, January 16, 1948

Louisiana State University, March 2, 1948

Tulane University, May 14, 1948

Southern California

Chairman: D. Geiger Secretary: A. J. Haagen-Smit Members: 67

Meetings: California Institute of Technology, November 4, 1947

University of Southern California, January 29, 1948

Scripps Metabolic Clinic, April 10, 1948

University of California, Los Angeles, June 10, 1948

Southwestern

Chairman: A. A. Hellbaum Secretary: R. W. Lackey Members: 84
 Meetings: Southwestern Medical College, November 15, 1947
 University of Texas, May 7, 1948

Western New York

Chairman: A. Dounce Secretary: S. L. Vaughan Members: 80
 Meetings: Cornell University, October 25, 1947
 Syracuse University, December 13, 1947
 University of Rochester, February 21, 1948
 University of Buffalo, April 24, 1948

Wisconsin

Chairman: W. H. McShan Secretary: D. M. Angevine Members: 59
 Meetings: University of Wisconsin, November 13, 1947

MEMBERSHIP

Members, March 31, 1947.....	2200		
Elected during year	138		
			2338
Resignations	16		
Arrears	4		
Deaths	19		
			39
Total Membership, March 31, 1948.....			2299
	Membership:	1938	1948
		1419	2299
Subscriptions, March 31, 1948.....			1492

Annual Report of the Treasurer

April 1, 1947-February 29, 1948

President Geiling appointed Doctors Opie, Cannan, Gregersen and Gold as Auditing Committee to examine the accounts of the Treasurer. This Committee requested Mr. Alexander Dolowitz, C.P.A., to go over the books of the Society and report to the Committee. The following is Mr. Dolowitz's abbreviated statement:

Society for Experimental Biology and Medicine
Statement of Assets and Liabilities
February 29, 1948

ASSETS		
Cash in banks		\$ 9,945.08
Investments:		
Surplus Fund (Special)	\$31,145.01	
Endowment Fund	23,093.84	54,238.85
Accounts receivable		1,428.80
Total assets		<u>\$65,612.73</u>
LIABILITIES		
Accounts payable	\$ 5,508.28	
Withholding taxes	132.60	
N. Y. Section	221.97	
Total liabilities		<u>\$ 5,862.85</u>
Net Worth		<u><u>\$59,749.88</u></u>
ENDOWMENT FUND		
N. Y. Title & Mortgage Co.		\$ 2,940.00
Title Guarantee & Trust Co.		114.01
Lawyers Mortgage Co.		74.43
Bowery Savings Bank		2,899.46
Century Federal Savings & Loan		80.75
U. S. Government Bonds		12,250.00
Industrial Bonds		1,848.62
Corn Exchange Bank		2,886.57
Total		<u><u>\$23,093.84</u></u>
SURPLUS FUND (Special)		
Harlem Savings Bank		\$ 1,474.65
U. S. Government Bonds		26,345.71
Industrial Bonds		2,726.69
Corn Exchange Bank		597.96
Total		<u><u>\$31,145.01</u></u>

Statement of Income and Disbursements

April 1, 1947 to February 29, 1948

INCOME

Dues	\$10,550.64	
N. Y. Section	204.25	
Subscriptions	\$12,251.38	
Reprints	3,794.74	
Space	1,195.32	
Cuts	447.05	
Changes	162.32	
Back Numbers	586.05	
	<hr/>	\$18,436.86
Interest—Special accounts	2.10	
Miscellaneous	10.74	
	<hr/>	
Total Income		\$29,204.59

DISBURSEMENTS

Salaries	\$ 3,778.07	
Office supplies, telephone and postage.....	1,680.15	
Printing	\$21,926.23	
Reprints	3,242.46	
Cuts	2,262.90	
Storage and insurance	75.80	
Refunds	8.50	
	<hr/>	\$27,515.89
Miscellaneous	741.29	
Paid to N. Y. Section	195.00	
	<hr/>	
Total disbursements		\$33,910.40
	<hr/>	
Excess of Disbursements over Income.....		\$ 4,705.81

MEMBERS' LIST

- Abramson, D. I. Maywood, Ill.
 Abramson, H. A. Coll. Phys. and Surg., N.Y.
 Abreu, B. E. Univ. of Cal. Med.
 Abt, Arthur F. Northwestern Univ.
 Acevedo, D. Univ. de San Marcos, Peru
 Adams, A. Elizabeth Mount Holyoke Coll.
 Adams, J. M. Univ. of Minn.
 Addis, Thomas Stanford Univ. Med.
 Adler, Harry F. Randolph Field, Texas
 Adlersberg, D. Beth Israel Hosp., N. Y.
 Adolph, E. F. Univ. of Rochester Med.
 Adolph, W. H. Yenching Univ., China
 Adriana, J. Louisiana State Univ.
 Albanese, A. A. N. Y. U. Med.
 Albaum, H. G. Brooklyn Coll.
 Alexander, Albert Mayo Foundation
 Alexander, Harry L. Washington Univ.
 Alexander, Hattie E. Babies Hosp., N. Y.
 Algire, G. H. National Cancer Inst.
 Allen, Bennet M. Univ. of Calif., L. A.
 Allen, Frank W. Univ. of Calif.
 Allen, J. G. Univ. of Chicago
 Allen, Shannon C. Oakland, Calif.
 Allen, Willard M. Washington Univ. Med.
 Allen, William F. Univ. of Oregon
 Alles, G. A. Univ. of Calif.
 Allison, J. B. Rutgers Univ.
 Almquist, H. J. Emeryville, Calif.
 Alt, Howard L. Northwestern Univ. Med.
 Althausen, T. L. Univ. of Calif. Med.
 Altschule, A. M. South. Reg. Res. Lab.,
 New Orleans
 Altschule, M. D. Waverly, Mass.
 Altshuler, S. S. El Paso, Texas
 Alvarez, Walter C. Mayo Clinic
 Alving, A. S. Univ. of Chicago
 Amberg, Samuel Mayo Clinic
 Amberson, W. R. Univ. of Maryland Med.
 Ambrose, A. M. West. Reg. Res. Lab.,
 Albany, Calif.
 Amoss, Harold L. Rockefeller Inst.
 Andersch, M. A. Univ. of Maryland Med.
 Anderson, Dorothy H. Coll. Phys. and Surg.,
 N. Y.
 Anderson, Evelyn Bethesda, Md.
 Anderson, H. H. Univ. of Calif. Med.
 Anderson, John A. Univ. of Utah Med.
 Anderson, John E. Univ. of Minn.
 Anderson, Richmond K. Rockefeller
 Foundation
 Yale Univ.
 Anderson, Rudolph J. Rockville, Conn.
 Anderson, William E. National Cancer Inst.
 Andervont, H. B.
- Andrew, Warren Southwestern Med.
 Andrus, E. C. Johns Hopkins Univ.
 Andrus, W. deW. Cornell Univ. Med. Coll.
 Angerer, C. A. Ohio State Univ.
 Angevine, D. M. Univ. Wisconsin Med.
 Anigstein, Ludwik Univ. of Texas Med.
 Annegers, John H. Northwestern Med.
 Ansbacher, Stefan Cincinnati, O.
 Antopol, William Newark, N. J.
 Apperly, Frank L. Med. Coll. of Va.
 Aring, C. D. Cincinnati Gen. Hosp.
 Armstrong, Charles Nat. Inst. of Health,
 Washington
 Armstrong, Charles D. Menlo Park, Calif.
 Armstrong, W. D. Univ. of Minn.
 Arnold, Lloyd Univ. of Ill.
 Arnow, L. E. Glenolden, Pa.
 Aron, H. C. S. Northwestern Univ. Med.
 Aronson, J. D. Henry Phipps Inst.
 Artom, Camillo Bowman Grey Med.
 Asdell, S. A. Cornell Univ.
 Asenjo, Conrad F. San Juan, Puerto Rico
 Ashby, W. M. Washington, D. C.
 Ashman, Richard Louisiana State Univ.
 Ashworth, C. T. Southwestern Med.
 Asmundson, V. S. Univ. of Calif.
 Atchley, D. W. Presbyterian Hosp., N. Y. C.
 Atkinson, William B. Coll. Phys. and Surg.,
 N. Y.
 Aub, Joseph C. Mass. Gen. Hosp., Boston
 Austin, J. Harold Univ. of Pa.
 Avery, B. F. Tehran, Iran
 Avery, O. T. Rockefeller Inst., N.Y.C.
 Avery, Roy C. Vanderbilt Univ.
 Aycock, W. L. Harvard Med.
 Ayo, C. Howard, R. I.
- Babkin, Boris P. McGill Univ.
 Bachem, Albert Univ. of Ill. Med. Coll.
 Baehr, George Mt. Sinai Hosp., N.Y.C.
 Baernstein, H. D. National Inst. of Health
 Bailey, Cameron V. N.Y. Post-Graduate Med.
 Bailey, Percival Univ. of Ill. Med.
 Baitsell, George A. Yale Univ.
 Baker, James A. N. Y. State Vet. Coll.
 Bakwin, Harry N. Y. Univ. Med. Coll.
 Baldwin, Francis M. Univ. of S. Calif.
 Baldwin, I. L. Univ. of Wisconsin
 Bale, William F. Univ. of Rochester
 Ball, G. H. Univ. of Calif., L. A.
 Ball, H. A. San Diego, Calif.

Balls, A. K.	West. Reg. Res. Lab., Albany, Calif.	Bergman, H. C.	Los Angeles, Calif.
Banerjee, S.	Calcutta, India	Berkman, Sam	Los Angeles, Calif.
Bang, Frederik B.	Johns Hopkins Hosp.	Bernhard, Adolph	Lenox Hill Hosp., N.Y.C.
Barach, Alvan L.	Coll. Phys. and Surg., N.Y.	Bernhart, F. W.	Mason, Mich.
Barber, W. Howard	New York Univ. Med.	Bernheimer, A. W.	N. Y. U. Med.
Bard, Philip	Johns Hopkins Univ.	Bernthal, T. G.	Med. Coll. of S. C.
Barer, Adelaide P.	State Univ. of Iowa	Berry, George P.	Univ. of Rochester Med.
Barker, S. B.	Iowa City, Ia.	Best, C. H.	Univ. of Toronto
Barnes, LeRoy L.	Cornell Univ.	Beutner, R.	Hahnemann Med. Coll., Philadelphia
Barnes, R. H.	Glenolden, Pa.	Bevelander, Gerrit	N. Y. U. Med.
Barnes, T. C.	Hahnemann Med.	Beyer, K. H., Jr.	Glenolden, Pa.
Barnett, George D.	Stanford Univ.	Biddulph, Clyde	Utah State Agric. Coll.
Barnett, Henry S.	Cornell Med. Coll.	Bier, Otto	Sao Paulo, Brazil
Barnum, C. J., Jr.	Univ. of Minn.	Bierman, W.	Mt. Sinai Hosp., N. Y.
Baronofsky, I. D.	Univ. of Minn.	Bieter, Raymond N.	Univ. of Minn.
Barr, David P.	Cornell Med. Coll.	Bills, C. E.	Mead, Johnson and Co., Evansville, Ind.
Barrett, Morris K.	Nat'l Cancer Inst.	Bing, Franklin C.	Am. Med. Assn., Chicago
Barron, E. S. G.	Univ. of Chicago	Bing, R. J.	Johns Hopkins Univ.
Barthley, S. H.	Dartmouth Coll.	Bird, Herbert R.	U. S. Dept. Agric.
Bass, Allan D.	Syracuse Univ. Med.	Birkhaug, Konrad E.	Geofysisk Inst., Bergen, Norway
Bass, Charles	Tulane Univ.	Birnbaum, G. L.	New York Med. Coll.
Bassett, D. L.	Stanford Univ.	Bishop, George H.	Washington Univ. Med.
Bast, T. H.	Univ. of Wisconsin	Biskind, G. R.	Mt. Zion Hosp., San Francisco
Bateman, J. B.	Frederick, Md.	Bittner, J. J.	Univ. of Minn. Med.
Bates, R. W.	New Brunswick, N. J.	Blair, Henry A.	Univ. of Rochester Med.
Batterman, R. C.	New York Univ. Med.	Blair, John E.	Hosp. for Joint Diseases, N.Y.
Bauer, J. H.	Rockefeller Fdtn., Paris	Blake, F. G.	Yale Univ.
Bauman, Louis	Presbyterian Hosp., N.Y.C.	Blalock, Alfred	Johns Hopkins Univ. Med.
Baumann, Carl A.	Univ. of Wisconsin	Blatherwick, Norman R.	Metropolitan Life Ins. Co., N. Y. City
Baumann, E. J.	Montefiore Hosp., N.Y.C.	Blinks, L. R.	Stanford Univ.
Baumberger, J. Percy	Stanford Univ.	Bloch, Robert G.	Univ. of Chicago
Baxter, James H.	Rockefeller Inst. Hosp.	Block, Richard J.	N. Y. Med. Coll.
Bayley, R. H.	Univ. of Okla. Med.	Block, Walter D.	Univ. of Michigan
Bayne-Jones, S.	Cornell Med. Coll.	Blood, Frank R.	Univ. of Denver
Bazett, H. C.	Univ. of Pa.	Bloom, William	Univ. of Chicago
Beach, Eliot F.	Detroit, Mich.	Bloomfield, A. L.	Stanford Univ. Med.
Beams, H. W.	State Univ. of Iowa	Bloor, W. R.	Univ. of Rochester
Bean, John W.	Univ. of Mich.	Blount, R. F.	Univ. of Texas Med.
Beard, J. W.	Duke Univ.	Blum, Harold F.	Princeton Univ.
Beck, Claude S.	Western Reserve Univ.	Blumberg, Harold	Richmond Hill, N. Y.
Becker, E. R.	Iowa State Coll.	Blumgart, H. L.	Beth Israel Hosp., Boston
Becker, R. F.	Washington Univ. Med.	Bodansky, A.	Hosp. for Joint Diseases, N. Y.
Beckman, Harry	Marquette Univ. Med.	Bodansky, Oscar	N. Y. U. Med.
Beeson, P. B.	Emory Univ.	Bodine, J. H.	State Univ. of Iowa
Behre, Jeannette A.	New York City	Bogen, Emil	Olive View, Calif.
Belding, David L.	Boston Univ.	Bollman, Jesse L.	Mayo Clinic
Bender, M. B.	Mt. Sinai Hosp., N. Y.	Bonnycastle, Desmond D.	Yale Med. School
Bengston, Ida A.	National Inst. of Health, Washington	Bondi, A., Jr.	Hahnemann Med. Coll.
Bennett, G. A.	Univ. of Illinois Med.	Booher, Lela E.	Minneapolis, Minn.
Bennett, L. L.	Univ. of Calif.	Boor, Alden K.	Univ. of Chicago
Berg, B. N.	Columbia Univ.	Boothby, Walter M.	Mayo Clinic, Rochester, Minn.
Berg, C. P.	State Univ. of Iowa		
Berg, William N.	New York City		
Bergeim, Olaf	Univ. of Ill.		

Carruthers, A.	Birmingham, England	Cohen, Milton B.	St. Alexis Hosp., Cleveland
Cartland, G. F.	Kalamazoo, Mich.	Cohen, P. P.	Univ. of Wis.
Cary, C. A.	U. S. Dept. of Agriculture	Cohn, A. E.	Rockefeller Inst., N. Y.
Casals, J.	Rockefeller Inst.	Cohn, Clarence	Michael Reese Hosp., Chicago
Casey, Albert E.	Birmingham, Ala.	Cohn, David J.	Monee, Ill.
Casida, L. E.	Univ. of Wisconsin	Cohn, Isidore	New Orleans, La.
Castaneda, M. R.	Hosp. General, Mexico City	Cole, Harold H.	Univ. of Calif., Davis
Castle, W. B.	Boston City Hosp.	Cole, Rufus I.	Rockefeller Inst., N. Y. City
Cattell, McKeen	Cornell Univ. Med. Coll.	Cole, Warren H.	Univ. of Ill. Med.
Cecil, R. L.	Cornell Univ. Med. Coll.	Cole, William H.	Rutgers Univ.
Cerecedo, L. R.	Fordham Univ.	Colien, F. E.	Akron, Ohio
Chadwicks, C. S.	Vanderbilt Univ.	Collens, William S.	Brooklyn, N. Y.
Chagas, Carlos	Univ. of Brazil	Collier, William D.	St. Elizabeth's Hosp.,
Chaikoff, I. L.	Univ. of Calif.		Youngstown, O.
Chambers, Robert	New York Univ.	Collings, W. D.	Univ. of Iowa Med.
Chambers, Wm. H.	Univ. of Maryland	Collins, D. A.	Temple Univ. Med.
Chang, Hsi Chun	Peiping Union Med. Coll.	Collip, J. B.	Univ. Western Ontario
Chang, Hsiao-Chien	Peiping Union Med. Coll.	Compere, E. L.	Northwestern Med.
Charipper, H. A.	New York Univ.	Conant, N. F.	Duke Univ.
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Venezuela—Jaffé W. G., Maier J., Pi-Suñer A.

AUTHORS' INDEX

(The numerals indicate the page.)

- Adriani, J., 520.
 Ahern, J. J., 625.
 Alexander, E., Jr., Woods, R. P., and Weiss, P. Nerve gap bridging. 380.
 Alles, G. A., 174.
 Ames, S. R., and Risley, H. A. Muscular dystrophy, aminoaciduria. 131.
 Anderson, J. C., 564.
 Armstrong, W. D., Schubert, J., and Lindenbaum, A. Carbon* distribution, body, excreta. 233.
 Atkinson, W. B., 537.
 Baker, B. L. Metrial gland, pregnancy, lactation. 492.
 Baker, W. B., 101.
 Ballin, H. M., 540.
 Barnes, R. H., 498.
 Barrows, A. D., 286.
 Batson, H. C., 534.
 Beard, D., 308, 309.
 Beard, J. W., 308, 309, 442.
 Beaver, D. C., 501.
 Bennett, A., 97.
 Bennett, L. L., Garcia, J. F., and Li, C. H. Hormone adrenocorticotrophic, growth, ketonemia. 349, 651.
 Bennett, L. L., Li, C. H., and Laundrie, B. Hypophysectomy, N retention, growth hormone. 94.
 Benson, W. M. Choline inhibition, adrenalin. 598.
 Berkman, S., Henry R. J., Housewright, R. D., and Henry, J. Streptomycin, bacterial adsorption. 65.
 Bigelow, R. R., 473.
 Bishop, L. K., 70, 622.
 Blair, H. A., 304.
 Bloch, R. G., 250, 625.
 Bloor, W. R., 332.
 Boche, R. D., 279.
 Boger, W. P., Baker, W. B., and Wilson, W. W. Penicillin, cerebrospinal. 101.
 Bond, G. C., Vanderbrook, M. J., Wiley, J. L., and Nook, M. A. Bacitracin, oral. 395.
 Bosshardt, D. K., 498.
 Bowman, D. E. Trypsin, hypotensive activity. 358.
 Boyles, P. W., 246.
 Briggs, G. M., 564.
 Brown, R. W., 216.
 Brown, S. O., Wilkening, M. C., and Schweigert, B. S. Tryptophan deficiency. 672.
 Brueckner, A. L., 293.
 Brumfield, H. P., Stulberg, C. S., and Halvorson, H. O. Virus, poliomyelitis purification—methanol. 410, 282.
 Bruner, H. D., Boche, R. D., Gibbon, M. H., and McCarthy, M. D., Phosgene poisoning, vagotomy, ECG. 279.
 Bruzzzone, S., and Lopez, H. Hormone, pregnenolones, cortical action. 578, 579.
 Buchanan, A. R., Roberts, J. E., and Robinson, B. E. Ergotoxine hyper-, hypothermia. 143.
 Budy, A. M., 324.
 Bullock, F., 425.
 Bunde, C. A., and Lackey, R. W. Saccharine, no influence, sugar. 581.
 Calder, R. M. Nicotinic acid, anoxia. 642.
 Cameron, D. B., 646.
 Campbell, B., 82.
 Campbell, C. C., 559.
 Cantarow, A., 485.
 Canzanelli, A., 137.
 Cargill, W. H. Serum albumin, intrav., renal function. 189.
 Carrera, G. M., and Changus, G. W. Phosphatase, acid demonstration, *Endamoeba histolytica*. 610.
 Carroll, R. T., 110.
 Casey, A. E., 593.
 Chaikoff, I. L., Zilversmit, D. B., and Entenman, C. Plasma phospholipid turnover, diabetic dog. 6.
 Challek, W. S., Wiersma, C. A. G., and Alles, G. A. Synapse, blocking, protective actions amines, NH_4 compds. 174.
 Chambers, V. C., 436.
 Chang, N. C., and Hou, T. C. Kala Azar, cold hemagglutinin. 72.
 Chang, M. C. Ova development, transplanted, storage temp. 680.
 Changus, G. W., 610.
 Charipper, H. A., 527.
 Chen, C. H., 450.
 Chen, K. K., 350.
 Christensen, L. R., 179, 184.
 Clapper, W. E., and Heatherman, M. E. Sulfonamide induced resistance, a strep. 392.
 Clark, G. W. Liver extr., pyridoxine, choline methionine content. 525.
 Cleveland, A. S., 477.
 Cowgill, G. R., 58.
 Cooper, M. L., and Keller, H. M. *Shigella sonnei*, tissue invasion, gavage. 87.
 Coret, I. A. Dibenamine congener actions, sympathomimetic amines. 553.
 Cox, H. R., 612.
 Crocker, T. T., 9.
 Cryst, E. E., 653.
 Curnen, E. C., 198.
 D'Angelo, S. A., Gordon, A. S., and Charipper, H. A. Adrenal, starvation response. 527.
 Davis, W. D., Jr., and Mayerson, H. S. Hypertension, blood vol. sympathectomy. 117.
 De Boer, B. Thiopental hypnosis, diet. 260.
 Decherd, G., 463.
 De Koning, J., Krichesky, B., and Glass, S. J. 17-Ketosteroid excretion, extrone administration, 322; 17-Ketosteroid excretion ovariectomized rabbits. 320.
 DeLoach, A. W., 120.
 Delson, B., Lubin, S., and Reynolds, S. R. M. Estrogens, ovarian spiral arteries. 96.